p14ARF Hypermethylation Is Common but *INK4a-ARF* Locus or *p53* Mutations Are Rare in Merkel Cell Carcinoma

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Although the exact molecular mechanisms of Merkel cell carcinoma (MCC) tumorigenesis are unknown, they likely involve complex genetic alterations and mutations similar to those seen in many other cancers. In this study, we obtained MCCs from 21 elderly patients (19 women, 2 men) and analyzed their DNA for mutation of exons of interest in several tumor-suppressor genes or oncogenes known to be frequently mutated in skin cancer: *p53* (exons 4–8), *Ras* (exons 1 and 2), *c-Kit* (exon 11), and the *INK4a-ARF* locus (encoding p14 and p16) (exons 1 and 2). Direct sequence analysis revealed *p53* mutations (that is, at codons 224, 234, and 294) in three tumors (14%) and *p16INK4a* mutations (that is, at codon 6) in one (5%). No mutations were detected in *Ha-Ras*, *Ki-Ras*, *N-Ras*, *c-Kit*, or *p14ARF*. On the other hand, methylation-specific PCR revealed methylation of p*14ARF* promoter DNA in eight of 19 analyzable tumor samples (42%) and *p16INK4a* promoter DNA in one of 19 analyzable tumor samples (5%). Together, these findings suggest that p*14ARF* silencing may be an important mechanism in MCC tumorigenesis, and thus a potential target for therapeutic intervention in this highly aggressive tumor type.

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INTRODUCTION

Merkel cell carcinoma (MCC) (also known as trabecular carcinoma, small-cell carcinoma of the skin, and primary cutaneous neuroendocrine carcinoma) is a rare but very aggressive skin cancer that occurs predominantly on sun-exposed body parts of elderly people (Taylor *et al.*, 2005). In the United States, the estimated incidence is 0.34 cases per 100,000 person years. MCC is assumed to arise from Merkel cells, epithelial-like neuroendocrine cells found in the epidermis, hair follicles, and mucosa. The prognosis for patients with MCC is poor; depending on the stage, the overall 5-year survival rate ranges between 25 and 75% (Agelli and Clegg, 2003; Suarez *et al.*, 2004). UV radiation is thought to be a principal cause of MCC, although its exact role in this regard is not clear. It may be that UV exposure exerts a combination of tumor-initiating, tumor-promoting, and/or immunosuppressive effects (cited in Kreimer-Erlacher *et al.*, 2003). It may be that an increase in Merkel cell density in chronically sun-exposed body parts raises the overall risk of developing MCC (Moll *et al.*, 1990). In any case, the molecular alterations underlying the development and progression of MCC are poorly understood; indeed, unlike the case for other nonmelanoma skin cancers (for example, squamous cell carcinoma (SCC) and basal cell carcinoma), in which the tumor-suppressor gene *p53* (Ziegler *et al.*, 1993; Agar *et al.*, 2004) or the *patched* gene (Hahn *et al.*, 1996; Johnson *et al.*, 1996; Heitzer *et al.*, 2007) have been identified as the major players, the primary gene responsible for MCC tumorigenesis has not yet been identified.

Despite this gap in knowledge, we speculated that the tumorigenesis of MCC, like that of many other cancers, results in part from the concerted actions of groups of genes. For instance, malignant melanoma, which in many respects is very similar to MCC, has a complex mutational profile, which involves alterations in *BRAF* (Daniotti *et al.*, 2004), *Ras* (Bos, 1989), *p53* (Ziegler *et al.*, 1993), *c-Kit* (Willmore-Payne *et al.*, 2005), and the *INK4a-ARF* locus (Pollock and Hayward, 1996). More recently, research on epigenetic mechanisms of cancer such as altered gene methylation status or histone modification has shed other light on MCC tumorigenesis. Aberrant DNA methylation is now thought to be one of the most common molecular alterations in many cancer cell types (Esteller, 2005). For instance, molecular genetic studies have revealed that the *p16INK4a* gene is frequently inactivated by 5′-CpG hypermethylation in primary central nervous system lymphoma (Nakamura *et al.*, 2001) and cutaneous SCC (Brown *et al.*, 2004), and that the human *p14ARF* promoter is aberrantly methylated in gliomas (Nakamura *et al.*, 2001), colorectal adenomas and carcinomas (Esteller *et al.*, 2000), esophageal

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Abbreviations: MCC, Merkel cell carcinoma; MSPCR, methylation-specific PCR; SCC, squamous cell carcinoma; SNP, single nucleotide polymorphism

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carcinomas (Xing *et al.*, 1999), and cutaneous SCCs (Brown *et al.*, 2004).

In this study, we obtained DNA samples from 21 MCCs and analyzed them for the presence of gene mutations commonly found in other forms of skin cancer, namely, *p53* (exons 4–8); *Ha-*, *Ki-*, and *N-Ras* (exons 1 and 2); *c-Kit* (exon 11); and the *INK4a-ARF* locus, which encodes *p16INK4a* and *p14ARF* (exons 1 and 2). We also examined the methylation status of the promoter regions of the *INK4a-ARF* locus by methylation-specific PCR (MSPCR) and the protein expression status of p16INK4a and p14ARF by immunohistochemical staining. As a result, we found that in our sampling of MCCs, all genes of interest were rarely mutated, but methylation of *p14ARF* was very common.

RESULTS

p53 and *INK4a-ARF* locus mutations are rare and *Ras* and *c-Kit* mutations are absent in MCC

DNA sequence analysis of 21 primary MCCs revealed a total of three *p53* mutations in three cases (14%) and one *INK4a-ARF* mutation (affecting p16 protein expression) in one case (5%) (Table 1). No mutations at all were detected in *Ha-*, *Ki-*, or *N-Ras*, or in *c-Kit* (exon 11). Concerning *p53*, we detected two mutations affecting the amino-acid sequence (that is, a T:A→A:A transversion in tumor 6 (exon 7, codon 234) and a frameshift-inducing deletion of G in tumor 13 (exon 8, codon 294)), and another mutation causing a silent G:C→A:T transition (that is, UV-type mutation) in tumor 19 (exon 6, codon 224) (Figure 1). All *p53* mutations that we detected occurred in the highly conserved middle region of *p53*,

Table 1. Results of sequencing analysis

	***p53*[1] (exons 5–8)**				***INK4a-ARF*[2] (exons 1+2)**				***Ha-*, *Ki-*, and *N-Ras*[3] (exons 1+2)**			
	Mutation				**Mutation**				**Mutation**			
T	**Site**	**Base change**	**aa change**	**SNP[4]**	**Site**	**Base change**	**aa change**	**SNP[5]**	**Site**	**Base change**	**aa change**	**SNP[6]**
1	None			None	None			None	None			None
2	None			Codon 72[7]	None			None	None			None
3	None			None	C42G	ga**C**→ga**G**	D14E	None	None			Codon 27[8]
4	None			Codon 72[7]	None			None	NR			NR
5	None			None	None			None	None			Codon 27[8]
6	T700A	**T**ac→**A**ac	Y234N	Codon 72[7]	None			None	None			None
7	None			None	None			None	None			None
8	None			None	None			None	NR			NR
9	None			None	None			Codon 148[7]	None			Codon 27[8]
10	None			None	None			None	None			Codon 27[7]
11	None			Codon 72[7]	None			None	None			None
12	None			Codon 72[7]	None			None	None			None
13	880delG	**G**ag→−ag	E294 fs	None	None			None	None			None
14	None			Codon 72[7]	None			None	None			Codon 27[7]
15	None			None	None			Codon 148[7]	None			Codon 27[7]
16	None			None	None			None	None			None
17	None			None	None			Codon 148[7]	None			Codon 27[8]
18	None			Codon 72[7]	None			None	None			None
19	G672A	ga**G**→ga**A**	E224E	Codon 72[8]	None			None	None			None
20	None			Codon 72[7]	None			None	None			None
21	None			None	None			None	None			None

aa, amino acid; NR, no result; SNP, single nucleotide polymorphism; T, tumor.
[1]Sequence is from GenBank entry X54156. Bold faced letters represent affected nucleotides.
[2]Sequence is from GenBank entry NM_000077. Bold faced letters represent affected nucleotides.
[3]Sequence for Ha-*Ras* is from GenBank entry NM_005343; sequence for Ki-*Ras* is from GenBank entry NM_033360; and sequence for N-*Ras* is from GenBank entry AF493919.
[4]Base change: GC; amino-acid change: arginine→proline; database of single nucleotide polymorphisms (ds SNP; available at: http://www.ncbi.nlm.nih.gov/SNP), Build ID: rs 1042522.
[5]Base change: G→A; amino-acid change: threonine to alanine; ds SNP, Build ID: rs3731249.
[6]Base change at Ha-*Ras*: T→C; amino-acid change: none; ds SNP, Build ID: rs12628.
[7]Heterozygous polymorphism.
[8]Homozygous polymorphism.

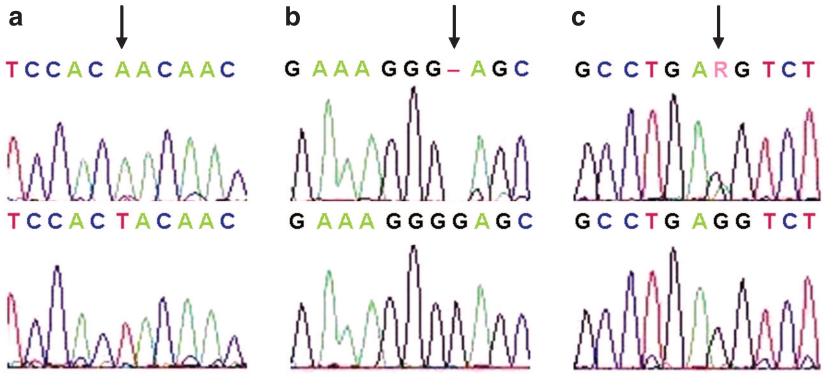

Figure 1. *p53* DNA sequencing results. Upper panels show electropherograms of MCCs that exhibited mutations: (**a**) tumor 6 (**b**) tumor 13, and (**c**) tumor 19. Lower panels show the electropherograms of corresponding tumor-adjacent normal skin (wild-type DNA). Arrows indicate (**a**) a homozygous T:A→A:A transversion in tumor 6 (exon 7, codon 234), (**b**) a homozygous deletion of G in tumor 13 (exon 8, codon 294), and (**c**) a heterozygous G:C→A:T transition (that is, UV-type mutation) in tumor 19 (exon 6, codon 224). Note that the letter R in the labelling of the electropherogram indicates the presence of both G and A in the DNA sequence.

which is responsible for sequence-specific DNA binding (Walker and Levine, 1996). In addition, we found that nine of 21 MCCs (43%) carried a polymorphism at codon 72 (that is, a G→C transversion), leading, as previously described (Matlashewski *et al.*, 1987; Thomas *et al.*, 1999), to an amino-acid change from arginine to proline. One of those nine samples (tumor 19) was homozygous for this *p53* polymorphism. Concerning the *INK4a-ARF* locus, we detected a C→G transition in tumor 3 at codon 6 (exon 1) (that is, a non-UV-type mutation), leading to an amino-acid change from aspartic acid to glutamic acid in p16INK4a, but no change in p14ARF. In addition, we found that three of 21 MCCs (14%) exhibited a common codon 148 polymorphism (exon 2), leading to the conversion of alanine to threonine (Lamperska *et al.*, 2002). Although we detected no mutations in the *Ras* genes, we did find that a previously described silent codon 27 polymorphism (that is, a T→C transition) (Hsieh *et al.*, 1994; Kreimer-Erlacher *et al.*, 2001) was present in seven of 19 analyzable MCCs (37%) (two of the original 21 MCCs were not analyzable because we could not amplify their DNA in the area surrounding codon 27) (Table 1). Of those seven samples, three were heterozygous and four were homozygous for this *Ras* polymorphism.

p14ARF promoter methylation is common in MCC

MSPCR-based analysis of 19 analyzable MCCs (for each gene) revealed the presence of methylated DNA at the *p14ARF* promoter in eight cases (42%) and at the *p16INK4a* promoter in one (5%) case (Table 2). Representative electrophoretic gels of MSPCR-amplified *p14ARF* and *p16INK4a* are shown in Figures 2 and 3. However, all samples containing methylated DNA were also shown to contain unmethylated DNA. (This allowed several possible and alternative inferences to be made about the tumor cell populations, that is, that they were only partially methylated, genetically diverse, methylated but in a non-biallelic manner, or contaminated by (umethylated) normal tissue cells; Brown *et al.*, 2004.) In four cases, the DNA for promoters *p14ARF* (tumors 7 and 15) and *p16INK4a* (tumors 8 and 12) could not be amplified enough to determine their methylation status, and therefore was not included in the determination of gene methylation rates.

To correlate *p16INK4a* and *p14ARF* DNA methylation status with protein expression status, we performed an immunohistochemical analysis. Representative photographs of cells stained to detect p16INK4a and p14ARF are shown in Figure 4, and a summary of the results is shown in Table 2. All 21 MCC samples showed p16INK4a staining and in most cases a high percentage of positive nuclei. Fourteen of the samples were also analyzable for p14ARF expression. Of those, only six (43%) showed p14ARF expression. More importantly, patchy staining (that is, p14ARF-positive cells interspersed among p14ARF-negative cells) was observed in those MCC samples, in particular being tumors 8, 11, and 17. Because of initial difficulties in establishing the p14ARF staining procedure and consumption of tissue, we were ultimately unable in seven cases to gather enough tissue for the staining of p14ARF and analysis of its expression.

DISCUSSION

In this study, we found that promoter hypermethylation of the *INK4a-ARF* locus was common in MCC. MSPCR analysis revealed the presence of methylated DNA at the *p14ARF* promoter in eight (42%) and *p16INK4a* promoter in one (5%) of 19 analyzable MCC samples (Figures 2 and 3; Table 2). This observation sheds important light on the pathogenesis of MCC because DNA methylation in promoter regions helps to regulate the gene silencing and expression, which occurs during the pathogenesis of many cancers including MCC. In particular, hypermethylation of CpG islands located in the promoter regions of tumor-suppressor genes such as *p16INK4a*, *BRCA1*, and *hMLH* is now established as an important mechanism of gene inactivation in cancer (Jones and Baylin, 2002; Esteller, 2005). The *INK4a-ARF* locus encodes two protein products (p16INK4a and p14ARF) that originate from identical exons 2 and 3 but different exon 1 (exon 1 is termed exon 1a in the case of p16INK4a and exon 1b in the case of p14ARF), and whose respective mRNAs are generated from separate promoters. Nonetheless, both proteins are so crucial to regulation of the cell cycle (Serrano

Table 2. Results of immunohistochemical staining of p16INK4a/p14ARF and MSPCR analysis of *INK4a-ARF* locus

	p16INK4a		p14ARF	
Tumor	Immuno-positivity[1]	Promoter methylation status[2]	Immuno-positivity[1]	Promoter methylation status[2]
1	+++[3]	–	++++[3]	–
2	++++	–	++++	–
3	++++	–	NA	±
4	++++	–	–	±
5	++++	–	NA	–
6	+++	–	–	±
7	++++	–	–	NR
8	++++	NR	+++	–
9	++	–	–	–
10	++++	–	–	–
11	++++	–	++	±
12	++++	NR	++++	±
13	++++	–	NA	±
14	++++	–	–	±
15	+	–	NA	NR
16	++++	±	–	–
17	++++	–	+++	±
18	++++	–	NA	–
19	++++	–	NA	–
20	++++	–	NA	–
21	++	–	–	–
All	–, 0	–, 18	–, 8	–, 11
	+, 1	±, 1	+, 0	±, 8
	++, 2	NR, 2	++, 1	NR, 2
	+++, 2		+++, 2	
	++++, 16		++++, 3	
			NA, 7	

NA, not analyzed; NR, no result; MSPCR, methylation-specific PCR.
[1]Nuclear positivity on immunohistochemical staining.
[2]–, unmethylated; ±, methylated and unmethylated; +, methylated.
[3]Percentage of nuclei positive: –, none; +, 0–10 %; ++, 11–40%; +++, 41–70%; ++++, 71–100%.

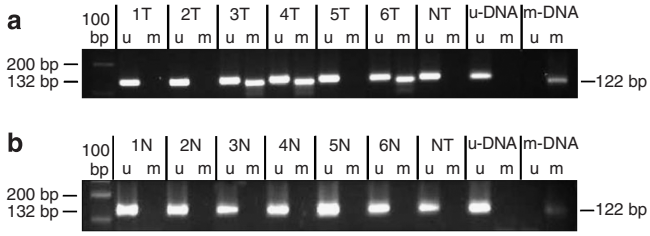

Figure 2. MSPCR analysis of *p14ARF* promoter region. Shown are representative MSPCR results from (**a**) tumors 1–6 (1T–6T) and (**b**) corresponding tumor-adjacent normal skin samples (1N–6N). Extracts were amplified by primers specific for methylated (m) or unmethylated (u) DNA, leading to fragment sizes of 122 bp (m) and 132 bp (u), respectively. CpG universally methylated DNA (m-DNA) and unmethylated DNA (u-DNA) were used to control for the specificity of MSPCR. NT, normal tissue DNA obtained from extraction of placental tissue. Note that the samples from tumors 1, 2, and 5 (1T, 2T, and 5T) contained only unmethylated, whereas those from tumors 3, 4, and 6 (3T, 4T, and 6T) contained both methylated and unmethylated DNA. Corresponding tumor-adjacent tissues and NT contained only unmethylated DNA.

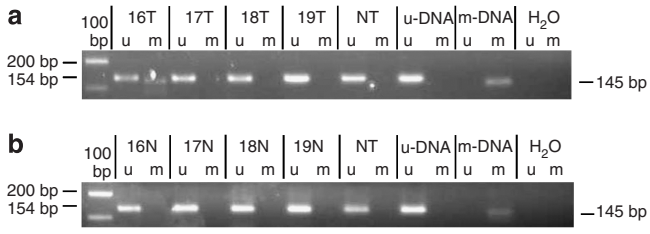

Figure 3. MSPCR of *p16INK4a* promoter region. Shown are representative results from (**a**) tumors 16–19 (16T–19T) and (**b**) corresponding tumor-adjacent normal skin samples (16N–19N). Extracts were amplified by primers specific for methylated (m) or unmethylated (u) DNA, leading to fragment sizes of 145 bp (m) and 154 bp (u), respectively. CpG universally methylated DNA (m-DNA) and unmethylated DNA (u-DNA) were used to control for the specificity of MSPCR. NT, normal tissue DNA obtained from extraction of placental tissue. H_20 served as negative control. Note that tumors 17–19 (17T–19T) contained only unmethylated, whereas tumor 16 (16T) contained both methylated and unmethylated DNA. The corresponding tumor-adjacent tissue and NT showed only unmethylated DNA.

et al., 1993; Brown *et al.*, 2004) that the functional loss of either one may lead to the unrestrained cell cycling and uncontrolled cell growth that is characteristic of the carcinogenic process. Several studies have also shown that methylated CpG sites are more sensitive to UV light (Tommasi *et al.*, 1997; Ikehata and Ono, 2006) and benzo[a]pyrene (Denissenko *et al.*, 1997; Chen *et al.*, 1998), possibly because their DNA repair mechanisms are more convoluted and impaired (Denissenko et al., 1997).

The methylation status of the *INK4a-ARF* locus may vary depending on tumor type, and different tumor types may have specific hypermethylation patterns (Iida *et al.*, 2000; Kawakami *et al.*, 2000; Kang *et al.*, 2001; To *et al.*, 2002; Brown *et al.*, 2004; Ishida *et al.*, 2005; Inda *et al.*, 2006). Our present observations of frequent *p14ARF* promoter hypermethylation and rare *p16INK4a* promoter hypermethylation are consistent with previous studies of colorectal carcinomas (Esteller *et al.*, 2000) and cutaneous SCCs (Brown *et al.*, 2004), indicating that these two promoters can be methylated independently. In their study of colorectal carcinomas, Esteller *et al.* (2000) found simultaneous absence of methylation in both promoters in approximately half of the cases, but after this, *p16INK4a* methylated alone, *p14ARF* methylated alone, and both methylated at similar rates. Importantly, *p15INK4b* (which is located a mere 14 kb upstream of *p14ARF*) was not found to be methylated at all.

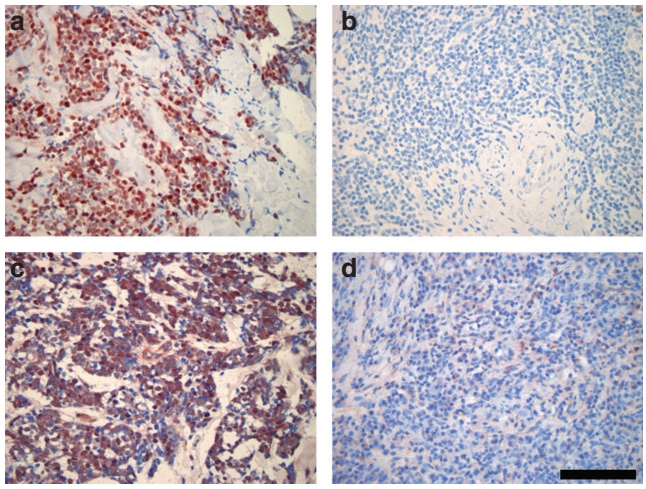

Figure 4. p16INK4a and p14ARF immunohistochemical staining. (**a**, **b**) Shown are representative results of p16INK4a staining of tumors 20 (**a**) and 15 (**b**). Note that p16INK4a nuclear immunopositivity was present in the majority of tumor cells in tumor 20, but was totally absent from this field of tumor 15. (**c**, **d**) Shown are representative results of p14ARF staining of tumors 2 (**c**) and 4 (**d**). Note that p14ARF nuclear immunopositivity was present in the majority of tumor cells in tumor 2, but only in a few cells in tumor 4. Sections were counterstained with hematoxylin. Bar = 0.1 mm.

In their study of cutaneous SCCs, Brown *et al.* (2004) noted *p16INK4a* and *p14ARF* promoter hypermethylation in 36% (13 of 36) and 42% (16 of 38) of cases, respectively. They also noted that in their tumor sample set, hypermethylation of both promoters was much less frequent than the presence together of hypermethylated *p16INK4a* and unmethylated *p14ARF*, or unmethylated *p16INK4a* and hypermethylated *p14ARF* (10 *vs* 70 *vs* 75%, respectively). In contrast, hypermethylation of both promoters appears to occur much more frequently in oral SCCs (Ishida *et al.*, 2005) and breast cancers (Silva *et al.*, 2003).

Our immunohistochemical analysis did reveal a good correlation between *INK4a-ARF* promoter methylation status and the expression of p16INK4a but not p14ARF protein (Table 2). All MCC samples showed p16INK4a positivity, but only one (tumor 16) exhibited promoter methylation. Concerning p14ARF, immunohistochemical results from six of eight promotor-methylated and seven of 11 promotor-unmethylated MCC samples were available for comparative correlation. In the six MCC samples that exhibited *p14ARF* promoter hypermethylation, p14ARF protein expression was detected in three of samples (tumors 11, 12, and 17), but not in the other three samples (tumors 4, 6, and 14). One possible explanation for this discrepancy is that both methylated and unmethylated DNA were present in those cases. On the other hand, in the seven MCC samples in which the *p14ARF* promoter was unmethylated, p14ARF protein expression was detected as expected in three samples (tumors 1, 2, and 8) but was unexpectedly undetectable in the remaining four samples (tumors 9, 10, 16, and 21). Our findings of patchy p14ARF staining and the mixture of positively and negatively stained cells within the MCCs that we examined are consistent with similar observations made in cutaneous SCCs (Brown *et al.*, 2004). In any case, this heterogenous immunohistochemical p14ARF staining is consistent with our ability to detect additional unmethylated DNA in any of the promoter-methylated samples (Table 2; Figure 2).

Regarding the mutational status of the *INK4a-ARF* locus in the 21 MCC samples, we observed only one missense mutation (5%) in codon 6 of exon 1 (the affected protein was p16INK4a), and three instances (14%) of polymorphism at codon 148 of exon 2. This observed prevalence of codon 148 polymorphism, a base change that results in the conversion of alanine to threonine and also happens to be the most frequently reported polymorphism in the *INK4a-ARF* gene (Lamperska *et al.*, 2002), was consistent with previous estimates of 12–14% (Fargnoli *et al.*, 1998; Holland *et al.*, 1999).

As indicated by previous studies of the regulatory role of the *INK4a-ARF* locus in p14ARF/p53 signaling pathways, *p53* mutations may be rarer in tumors in which this locus is inactivated than in tumors that express wild-type *INK4a-ARF* (Pomerantz *et al.*, 1998). This would be consistent with the low rate of *p53* mutation and high rate of *p14ARF* hypermethylation that we observed in this study. However, 43% (9 of 21) of the MCCs we analyzed also exhibited a previously described polymorphism at codon 72 of the *p53* gene (G→C transversion) (Matlashewski *et al.*, 1987; Thomas *et al.*, 1999). This *p53* polymorphism, which lies in a region critical for apoptotic signalling, leads to an amino-acid change from arginine to proline, which alters the biochemical and functional properties of the p53 protein (Matlashewski *et al.*, 1987; Thomas *et al.*, 1999).

Whether this polymorphic alteration has consequences for skin cancer tumorigenesis, however, remains a controversial issue (McGregor *et al.*, 2002; Chen *et al.*, 2003; Manson *et al.*, 2004). Although our present findings are limited by the lack of a control population and so do not allow us to draw any definite conclusions, we can conclude in light of these other studies that MCC risk is probably not influenced by the prevalence of the *p53* codon 72 polymorphism.

No Ha-, Ki-, or N-*Ras* mutations were detected in our study, even at codons 12, 13, and 61, where functionally relevant mutations often do occur (Popp *et al.*, 2002). These results confirm and extend the work of Popp *et al.* (2002), who detected no *Ras* mutations in six MCC cell lines. However, in over a third of the MCCs (37% (7 of 19)) that we examined in this study, we detected a silent polymorphism (that is, a T→C transition in codon 27 of the Ha-*Ras* gene) that has been previously described and implicated in skin cancer development by others (Hsieh *et al.*, 1994; Kreimer-Erlacher *et al.*, 2001). Although it was difficult until very recently to understand how a silent (synonymous) gene polymorphism might increase tumor risk, work by Kimchi-Sarfaty *et al.* (2007) suggests how this might occur. In brief, they have demonstrated that the shape of a protein is determined by more than just its amino-acid sequence and that a single-nucleotide polymorphism may result in a different protein conformation. This might occur via the effect of a single-nucleotide polymorphism on mRNA folding,

which in turn might affect the rate of mRNA translation and subsequent protein folding.

The two cell-cycle control pathways targeted most frequently during tumorigenesis are the pRB (pRb/p16INK4a/cyclin D1) and p53 (p14ARF/mdm2/p53) pathways, although the alterations in each pathway depend on tumor type. In the case of MCC, alterations in the p53 pathway appear to be most crucial, as suggested by the high frequency of *p14ARF* promoter methylation and low frequency of *p16INK4a* promoter methylation in our study. Consequently, this pathway might be targeted epigenetically with demethylating agents such as 5-aza-2/deoxycytidine (Carr *et al.*, 2006) or DNA methylation inhibitors such as zebularine [1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] (Yoo *et al.*, 2004). For instance, preclinical work by Carr *et al.* (2006) has shown that mRNA expression of *p14ARF* could be restored in methylated neuroblastoma cells (GIMEN) following treatment with 5-aza-2/deoxycytidine. In experimental studies involving transplantation of human bladder cancer cells into nude mice, Cheng *et al.* (2004) demonstrated that continuous administration of zebularine not only maintained *p16* gene expression, sustained demethylation, and prevented remethylation, but also induced global demethylation of various hypermethylated regions in the cells. In fact, therapeutically targeting methylation in MCC might be a much more efficacious strategy than targeting, for instance, the c-Kit tyrosine kinase pathway with kinase inhibitors such as imatinib mesylate. This is especially so in light of our inability to detect any (activating) c-Kit exon 11 mutations in MCC and recent work by Swick *et al.* (2007).

MATERIALS AND METHODS

Patients and tumors

Paraffin-embedded tissue samples of 21 primary MCCs archived at our institution were available for analysis. In all cases, the diagnoses were confirmed by immunohistochemical staining for neuroendocrine markers, such as neuron-specific enolase or chromogranin A, and/or cytokeratin 20. The MCCs were obtained from an elderly and predominantly female population of 21 patients (19 women, 2 men; mean age (range) at diagnosis, 78.5 (65–91) years). Lesion sites in these cases were the head ($n=12$), trunk ($n=2$), arm ($n=4$), leg ($n=2$), and an unknown lesion site ($n=1$). The study protocol (application number 18–139 ex 06/07) was approved by the local Ethical Committee at the Medical University of Graz, Austria, and was conducted according to the Declaration of Helsinki Principles. All patients who were alive at the time of our study, provided informed consent to DNA analysis of the MCC samples.

DNA extraction

Six-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded tissue samples and mounted on slides. Next, the slide-mounted sections were deparaffinized by incubation in xylene for 10 minutes and in ethanol (100%) for another 10 minutes, and then air-dried. All samples were manually dissected to minimize non-tumor cell content. Next, each tissue sample was scraped off its slide, suspended in 200 µl of a lysis solution containing 0.1 M Tris-HCl (pH 8) and 1 µg µl^{-1} proteinase K, incubated for at least 48 hours at 56 °C, boiled for 10 minutes, purified using a QIAmp DNA Mini Kit (Qiagen, Vienna, Austria), and finally stored at −20 °C until analysis.

Table 3. Self-designed primer sequences for PCR and sequencing

Gene	Amplified region	5′→3′
Ki-*Ras*[1]	Exon 1 (codon 12/13) forward	ATTATAAGGCCTGCTGAAAATG
Ki-*Ras*[1]	Exon 1 (codon 12/13) reverse	TGAAAATGGTCAGAGAAACC
Ki-*Ras*[1]	Exon 2 (codon 61) forward	TCAGGTGCTTAGTGGCCATTT
Ki-*Ras*[1]	Exon 2 (codon 61) reverse	CAAAGAAAGCCCTCCCCAGTCCT
N-*Ras*[2]	Exon 1 (codon 12/13) forward	CAGGTTCTTGCTGGTGTGAAA
N-*Ras*[2]	Exon 2 (codon 61) reverse	CACAAAGATCATCCTTTCAGA

[1]Sequence is from GenBank entry NM_033360.
[2]Sequence is from GenBank entry AF493919.

PCR

The template DNA for PCR consisted of approximately 50 ng of tumor DNA in a 50-µl solution containing PCR-10 × buffer (100 mM Tris-HCl, (pH 8.3), 500 mM KCl), 1.5 mM $MgCl_2$, 200 µM of each deoxyribonucleoside triphosphate, 15 pmol (300 nM) of the upstream and downstream primers for the respective exons, and 2.5 U of AmpliTaq Gold Polymerase (Applied Biosystems, Vienna, Austria). The following PCR primer sequences were used: *c-Kit*, as described by Lasota *et al.* (1999) (2003); *p53*, *INK4a-ARF*, and *Ha-Ras* (exon 2) as designed by us (Kreimer-Erlacher *et al.*, 2001, 2003; Seidl *et al.*, 2001; Wolf *et al.*, 2004), *Ha-Ras* (exon 1) forward primer, as described by Albino *et al.* (1989), and reverse primer, as described by Popp *et al.* (2002); and *N-Ras* exon 1 reverse primer and *N-Ras* exon 2 forward primer, as described by Albino *et al.* (1989). The *Ki-Ras* exons 1 and 2, *N-Ras* exon 1 forward, and *N-Ras* exon 2 reverse primers (Table 3) were designed by us.

All reaction mixtures were subjected to 40 cycles of amplification in a thermocycler (MyCycler, Vienna, Austria). Before the first cycle, tubes containing PCR mixtures were incubated for either 12 minutes at 94 °C (*p53* exons 4–8; *Ha-*, *Ki-*, and *N-Ras* exons 1 and 2; and *c-Kit* exon 11) or 15 minutes at 95 °C (*INK4a-ARF* exons 1 and 2). Each cycle consisted of denaturation at either 94 °C for 45 seconds (*p53*; *Ha-*, *Ki-*, and *N-Ras*; and *c-Kit*) or 95 °C for 30 seconds (*INK4a-ARF*). Annealing was performed at either 60 °C for 30 seconds (*p53*; *Ha-*, *Ki-*, and *N-Ras*; and *c-Kit*) or 57 °C for 30 seconds (*INK4a-ARF*). Polymerization was performed at either 72 °C for 30 seconds (*p53*; *Ha-*, *Ki-*, and *N-Ras*; and *c-Kit*) or 72 °C for 60 seconds (*INK4a-ARF*). After the last amplification cycle, PCR mixtures were incubated for either 7 minutes at 72 °C (*p53*, *Ha-*, *Ki-*, and *N-Ras*; and *c-Kit*) or 10 minutes at 72 °C (*INK4a-ARF*). Tubes containing no template DNA were included in each PCR run as negative controls for potential contamination. All amplification products were then purified by gel electrophoresis on a 2.5% MetaPhor gel (Cambrex, Rockland, ME) and subsequent gel extraction (Wizard SV Gel and PCR clean-up system; Promega, Mannheim, Germany).

DNA sequencing

Thirty nanograms of each tumor's DNA were amplified by reaction with 3.2 pmol (160 nM) of primer for the different exons of the specific genes and sequencing reagents (Big Dye Terminator v1.1 Cycle Sequencing KIT; Applied Biosystems). The amplification products were then purified on spin post-reaction clean-up plates (Sigma-Aldrich, Vienna, Austria), and analyzed in an ABI 3130 genetic analyzer (Applied Biosystems). In each case, DNA from normal tissue was simultaneously analyzed to rule out PCR-generated mutations. Direct DNA sequencing was performed at least twice per tumor sample, and the presence of a mutation was always confirmed by sequencing the opposite DNA strand. Sequence analysis was done on a personal computer programmed with sequence analysis software (SeqScape; Applied Biosystems). The primers used for sequencing were identical to those described above for PCR.

MSPCR

MSPCR analysis was carried out, essentially, as described previously (Herman *et al.*, 1996). The method is based on the principle that treating DNA with sodium bisulfite results in the conversion of unmethylated cytosine residues into uracil. After such treatment, the sequence of originally methylated DNA will differ from that of originally unmethylated DNA and will then be distinguishable by sequence-specific PCR primers. Bisulfite modification of tissue DNA was conducted with the EpiTect Bisulfite KIT (Qiagen). To analyze the methylation status of both *p16INK4a* and *p14ARF* gene promoter regions, MSPCR was carried out using separate reaction mixtures containing primers specific for unmethylated and methylated DNA, respectively. For both *p16INK4a* and *p14ARF* MSPCR, bisulfite-modified universally methylated or unmethylated DNA (Chemicon International, Hampshire, United Kingdom) was always used as a positive control and distilled water as a negative control. *p16INK4a* MSPCRs were conducted using the CpGWIZ p16 Amplification Kit (Chemicon International) and Hot StaR Taq DNA polymerase (Qiagen).

All reaction mixtures were subjected to 40 cycles of amplification in a thermocycler as follows: an initial denaturation step of 95 °C for 15 minutes; repeated cycles of 95 °C for 45 seconds, 60 °C for 45 seconds, and 72 °C for 60 seconds; and a final extension step of 72 °C for 7 minutes. Bisulfite-treated DNA was amplified by reaction with primers specific for the methylated *p14ARF* sequence and the unmethylated *p14ARF* sequence, as previously described by Esteller *et al.* (2000), using Hot StaR *Taq* DNA-Polymerase (Qiagen). Again, all reaction mixtures were subjected to 40 cycles of amplification in a thermocycler as follows: an initial denaturation step of 95 °C for 15 minutes; repeated cycles of 95 °C for 45 seconds, 55 °C for 45 seconds, and 72 °C for 60 seconds; and a final extension step of 72 °C for 7 minutes. All PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Immunohistochemical staining

Four-micrometer-thick, slide-mounted tissue sections were deparaffinized in xylene and rehydrated in water. The slides were stored in a warming cupboard overnight at 80 °C. Immunohistochemical staining of p16INK4a was performed with a TechMate Horizon apparatus (DakoCytomation autostainer; DAKO, Vienna, Austria) according to the manufacturer's protocol (www.DakoCytomation.com), using a murine anti-human $p16^{INK4a}$ concentrate (DAKO) and biotinylated goat anti-mouse serum (DAKO) for detection. Negative control reagent (containing monoclonal mouse IgG2a antibody to *Aspergillus niger* glucose oxidase, an enzyme neither present nor inducible in mammalian tissue) (DAKO) was used instead of the primary antibody to exclude nonspecific p16INK4a staining. Immunohistochemical staining of p14ARF was performed as previously described (Brown *et al.*, 2004), except that antigens were retrieved by boiling in 10 mM citrate buffer (pH 6) for 13 minutes in a microwave oven (750 watts). Rabbit anti-human p14ARF antibody (p14ARF/p16β Ab-4; Lab Vision, Vienna, Austria) and biotinylated goat anti-rabbit serum (DAKO) were used for detection. Rabbit IgG (Sigma-Aldrich) was used instead of primary antibody to exclude nonspecific p14ARF staining. Reactions were developed using 3,3′-diaminobenzidine solution (DAKO) in the case of p16INK4a and AEC Chromogen (DAKO) in the case of p14ARF. Slides were counterstained with hematoxylin. Since both p14ARF and p16INK4a are nuclear proteins, only nuclear reactivity was considered a positive sign of protein expression. Internal positive controls included basal keratinocytes for p16INK4a and hair follicles and glandular structures for p14ARF. All immunohistochemical stainings were scored by two of the investigators (A Lassacher and P Wolf).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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