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p53 Abnormalities are rare events in neuroendocrine (Merkel cell) carcinoma of the skin

An immunohistochemical and SSCP analysis

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Abstract The aim of the present study was to assess a possible role of the tumour suppressor gene *p53* in neuroendocrine (Merkel cell) carcinoma of the skin with regard to tumour development and tumour progression. p53 was investigated in a series of routinely processed Merkel cell carcinomas, with application of four different p53 antibodies (CM-1, PAb1801, DO7, and PAb240) to 25 carcinomas and screening for *p53* mutations of exons 4–8 by single-strand conformation polymorphism (SSCP) analysis in 9 cases. All 25 tumours in the present series showed the characteristic microscopic and immunohistochemical features of Merkel cell carcinoma of the skin. In 5 of the 25 Merkel cell carcinomas investigated 5–10% of tumour cell nuclei showed a positive p53 reaction with at least one anti-p53 antibody. A few scattered p53 positive nuclei were found in an additional 9 cases. The remaining 11 cases completely lacked p53 immunostaining. SSCP analysis of exons 4–8 revealed no significant alterations in the mobility shift of the single strand DNAs in the five cases with 5–10% p53-immunoreactive tumour nuclei or in five cases lacking *p53* accumulation significant. Our results suggest that alterations of the *p53* gene play only a minor part in the development or progression of Merkel cell carcinoma of the skin.

Key words Carcinogenesis · Tumour development · Tumour progression

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Introduction

Neuroendocrine (Merkel cell) carcinoma of the skin represents an uncommon but well recognised clinicopathological entity, which was described by Toker in 1972 [35]. From ultrastructural and immunohistochemical studies Merkel cell carcinoma is thought to originate from cutaneous Merkel cells, although the histogenesis of the tumour is still not unambiguous [11, 34, 39]. The differential diagnosis of Merkel cell carcinoma includes metastatic carcinoma, especially small cell anaplastic carcinoma of the lung, malignant lymphoma, and poorly differentiated primary neoplasms of cutaneous origin.

The tumour suppressor gene *p53* encodes for a nuclear phosphoprotein, which in its wild-type form inhibits cell proliferation and transformation, particularly in cells with DNA damage [10, 17, 25]. Since *p53* mutations and protein alterations are the most common abnormalities identified in human cancer [20], *p53* has been considered as a marker of both malignancy and tumour progression. Mutations at the *p53* locus (17p13.1) lead in the majority of cases to synthesis of aberrant p53 protein (mutant form) with prolonged half-life and increased stability, but a variety of extra genetic events have also been found to stabilise the p53 protein product. This accumulated protein is the target of immunohistochemical p53 detection [16].

The aim of the present study was to investigate p53 in a series of routinely processed Merkel cell carcinomas on both the protein and the genetical level. For this purpose we used four different p53 antibodies (CM-1, PAb1801, DO7, and PAb240); subsequently SSCP analysis was performed in five tumours showing immunohistochemical p53 expression in more than 5% of tumour cell nuclei.

Materials and methods

Routinely formalin-fixed and paraffin-embedded tissues from 25 primary Merkel cell carcinomas of the skin were used in the pres-

Table 1 Source and optimal dilution of antibodies used

Antibody	Source	Dilution
Anti-p53 antibodies		
CM-1	Medac, Hamburg, Germany	1:20,000
Pab1801	Medac	1:400
Pab240	Medac	1:50
DO7	Medac	1:500
Chromogranin A	BioGenix, San Ramon, USA	1:800
Chromogranin B	R. Fischer-Colbrie Dept. of Pharmacology, University of Innsbruck, Austria	1:2,000
Secretogranin II	R. Fischer-Colbrie Dept. of Pharmacology, University of Innsbruck, Austria	1:2,000
Broad spectrum cytokeratin (Kl-1)	Dianova, Hamburg, Germany	1:100
Cytokeratin 20	Progen, Heidelberg, Germany	1:50
Synaptophysin	Boehringer, Mannheim, Germany	1:20
Neuron specific enolase	Immunotech, Marseilles, France	1:20
Epithelial membrane antigen	Dako, Copenhagen, Denmark	1:10

ent study; the age of the patients (16 women, 9 men) ranged from 50 to 89 years (mean 71 years). Seven patients developed a local recurrence and 1 patient, lymph node metastases; none of the patients died from tumour disease. The tumour size ranged from 0.5 cm to 2.9 cm. There were 15 located on the face or head and 10 on the shoulder or arm.

For immunohistochemical stains serial sections were cut at a thickness of 4 µm and mounted on poly-L-lysine coated glass slides. The following antibodies were used: Primary anti-p53 antibodies (source and dilution see Table 1): CM-1, a rabbit polyclonal antiserum raised against full-length human *p53*; PAb1801, a murine monoclonal antibody that recognises a denaturation-resistant epitope of p53 protein; PAb240, a murine monoclonal antibody that reacts with a conformation-dependent epitope in some *p53* mutants (designed originally for frozen sections; the antibody can be used on paraffin-embedded tissue after wet autoclave pretreatment [10, 11]); DO7, a murine monoclonal antibody that recognises both wild-type and mutant forms of p53.

In addition, all cases were incubated with antibodies against chromogranin A, chromogranin B, secretogranin II [5], broad spectrum cytokeratins, cytokeratin 20 [26], synaptophysin, neuron-specific enolase, and epithelial membrane antigen (source and dilution see Table 1).

The sections were dewaxed in xylene and rehydrated through a series of graded ethanol according to routine standards; subsequently the sections were rinsed in distilled water and 0.01 M TRIS-HCl buffer (pH 7.4). Prior to immunostaining with the various antibodies against p53, the sections were pretreated for antigen retrieval by the wet autoclave method [2]. Dewaxed sections were placed in sodium citrate buffer (0.01 M Na-citrate monohydrate, pH 6.0 [27]) containing plastic Coplin jars and heated for 10 min (for antibodies CM-1 and PAb1801) or 5 min (for antibodies DO7 and PAb240) at 120°C in a bench type Gössner Laborautoklav (GLA-42–2). Subsequent to autoclaving, slides were allowed to cool to room temperature over a period of approximately 30 min, followed by a brief rinse in 0.05 M TRIS-HCl buffer (pH 7.4). All other antibodies were applied without prior pretreatment. Incubation with the primary antibodies was carried out overnight (16–20 h) in a humidified chamber at 4°C.

Subsequent to primary antibody incubation a rabbit-anti-mouse or mouse-anti-rabbit bridging antibody (1:30 and 1:125 in PBS; 30 min at room temperature; Dako, Copenhagen, Denmark) and a monoclonal mouse or rabbit APAAP complex (1:100 in PBS; 60 min at room temperature; Dianova, Hamburg, Germany) were applied. For the PAb240 and DO7 antibodies a "double-APAAP" reaction was performed (a 10-min repeat for both the bridging antibody and the APAAP complex). The bridging antibodies and the APAAP complex were applied to a semi-automatic immunostaining device ("Omnibus"; Quartett, Berlin, Germany). Subsequently the enzyme reaction was developed for 25 min at room temperature in a freshly prepared new fuchsin solution containing naphthol-bi-as-phosphate. Finally the sections were lightly counterstained with haematoxylin and mounted in Kayser's glycerin gelatin.

Omission of primary antibodies and application of inappropriate antibodies were used as negative controls. A breast carcinoma case with a proven *p53* mutation resulting in p53 protein overexpression and colonic carcinomas, malignant melanomas of the skin, and carcinomas of the oral cavity (3 cases each) with substantial immunohistochemical p53 overexpression were used as positive controls.

DNA from the five cases showing more than 5% p53-positive tumour cell nuclei and from five cases completely lacking p53 protein accumulation was extracted from 5-µm thick paraffin sec-

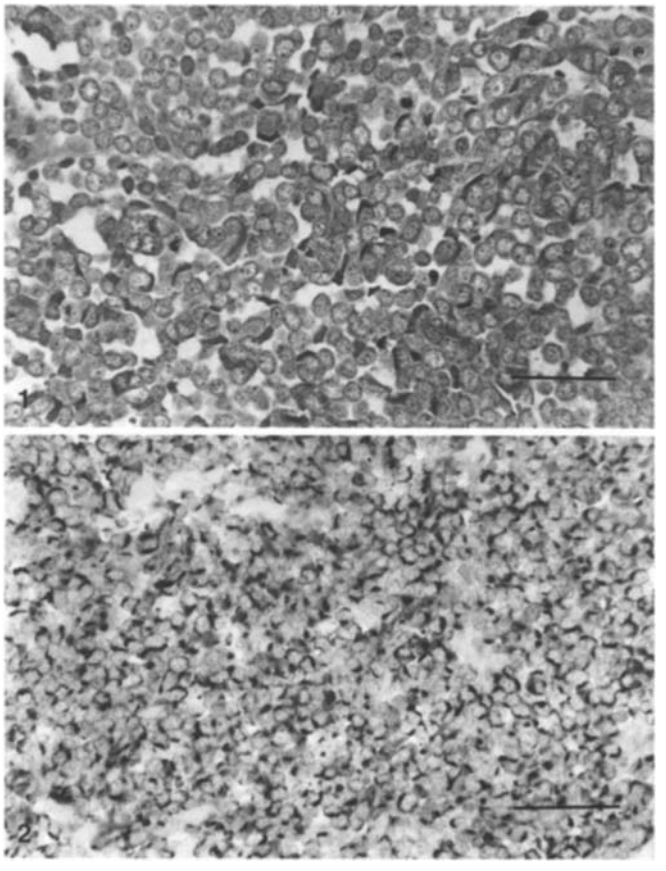

Fig. 1 Histological appearance of Merkel cell carcinoma. Typical morphology of neuroendocrine (Merkel cell) carcinoma of the skin with monomorphic tumour cells although several mitoses can be seen. H&E, *bar* 120 µm)

Fig. 2 Cytokeratin 20 expression in Merkel cell carcinoma. Merkel cell carcinoma exhibiting distinct dot-like paranuclear staining with cytokeratin 20. (APAAP technique, *bar* 250 µm)

tions. After removal of excess paraffin, proteinase K digestion was carried out overnight at 55°C. DNA was prepared by standard phenol/chloroform methods as described by Maniatis et al. [24]. Samples were stored in TRIS-HCl pH EDTA. Primers used for amplification and sequencing were synthesised by the phosphoramidite method using biotin-phosphoramidite for 5' biotinylated oligonucleotides by an automated oligonucleotide synthesiser (Genassembler, Pharmacia Biotech). All primers were used without further purification.

In the SSCP-PCR procedure, p53 exons 4–8 were amplified in five different PCR reactions. The oligonucleotide primers used have been described in detail elsewhere [8]. Each PCR reaction of a final volume of 20 µl contained: 40 ng of genomic DNA, 1 pmol/µl each of sense and antisense primers, each, 2.5 mM MgCl2 200 µM desoxynucleotide triphosphates, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.5 U Ampli-Taq polymerase (Perkin-Elmer Cetus). DNA was amplified following 40 cycles of PCR (30 s at 92°C, 90 s at 52–60°C, and 90 s at 72°C) using an automated thermocycler (Biomed).

Then 1 µl of the amplification mixture was added to 1.5 µl 95% deionized formamide heated to 94°C for 2 min and kept on ice until loading. After application of the samples to a 8% native ultrathin polyacrylamide gel baked on GelBond PagTM (FMC), electrophoresis was performed in a discontinuous buffer system with 35 mM sulphate-borate (pH 9.0) as the leading trailing ion and 141 mM Tris-borate (pH 9.0) as described by Allen et al. [1]. Gels were run on a horizontal electrophoresis system (Multiphor, Pharmacia Biotech) at 15°C and 2 mA for 5 to 16 h. Finally, bands were visualised by silver staining according to standard protocols [12].

Tissue from a healthy person was used for standard controls. Tumour tissues with known *p53* gene mutations in the respective exons (4–8) were used as controls to prove gel conditions able to recognise aberrantly migrating bands.

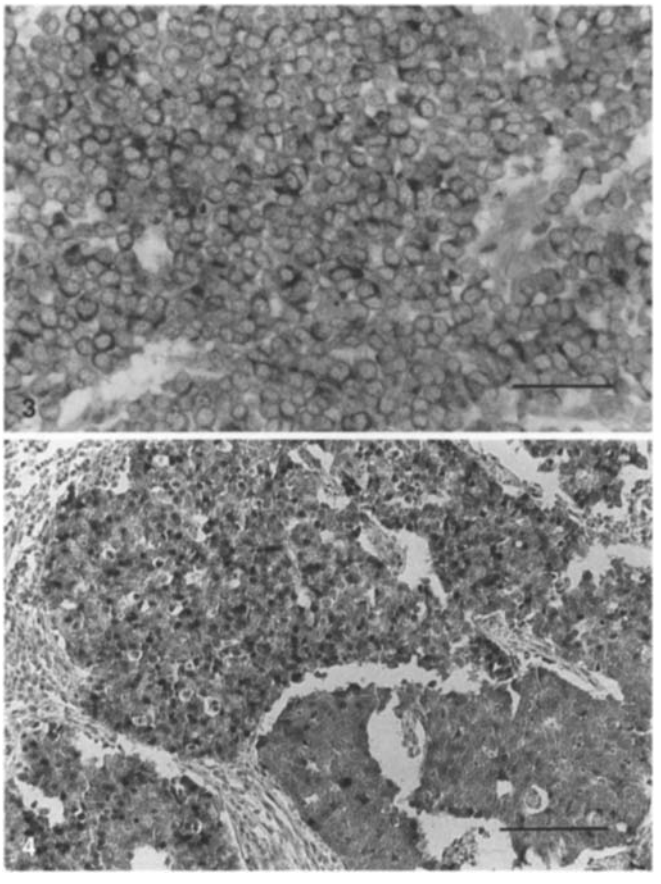

Fig. 3 Chromogranin B expression in Merkel cell carcinoma. Neuroendocrine differentiation of Merkel cell carcinoma demonstrated by chromogranin B immunoreactivity. PE-11 antibody, APAAP technique, *bar* 120 µm

Fig. 4 *p53* expression in Merkel cell carcinoma. Merkel cell carcinoma with an area showing approximately 10% p53 positive tumour cell nuclei; note focal p53 positivity. The slightly less well preserved morphology is due to the antigen retrieval procedure performed. CM-1, APAAP technique after wet autoclave pretreatment [10], *bar* 350 µm

Results

All tumours in the present series showed the characteristic microscopic features of Merkel cell carcinoma of the skin (Fig. 1). All were located in the dermis and exhibited a trabecular, solid, or diffuse growth pattern. The tumour cells were rather monomorphic, though mitoses were numerous. The results of immunohistochemical staining with the various neuroendocrine and epithelial markers are in agreement with findings reported in the literature (for review see ref. [5]) and confirmed the diagnosis of Merkel cell carcinoma; NSE, broad-spectrum cytokeratin, cytokeratin 20 (Fig. 2) and chromogranin B

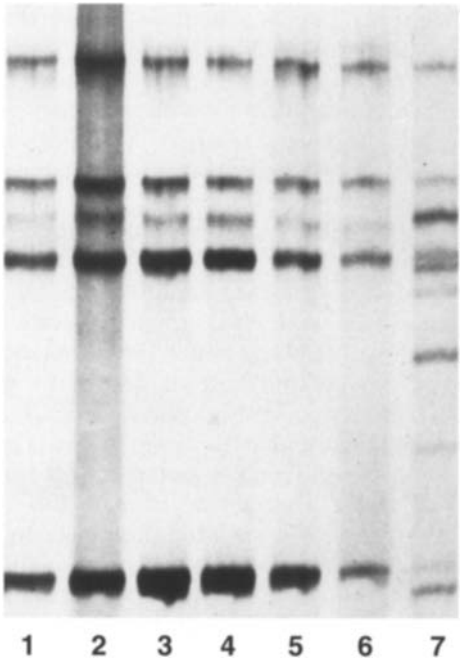

Fig. 5 SSCP analysis of exon 5 of the *p53* gene. Typical example of non-isotopic PCR-SSCP analysis of exon 5. Lane 7 depicts a malignant melanoma case with a known *p53* gene mutation in this exon; a normal control is shown in lane 1. In lanes 2–6 a normal SSCP pattern is shown in the five Merkel cell carcinomas which showed immunohistochemically 5–10% *p53*-positive tumour cell nuclei, suggesting that no mutational alterations have occurred in the respective target sequences of those cases (Note that SSCP analysis of exon 5 reveals a pattern of three bands owing to two possible conformations of one of the two single strands with a predominant conformation resulting in one stronger and one weaker band in PAGE. Bands in the lower part of the gel correspond to undenatured double-stranded DNA)

(Fig. 3) were found in all tumours. Chromogranin A and EMA were detectable in 18 cases, secretoneurin in 8 cases, and synaptophysin in 13 cases.

In 5 of the 25 Merkel cell carcinomas investigated 5–10% of tumour cell nuclei showed a positive *p53* reaction with at least one of the anti-*p53* antibodies recognising the p53 gene product in its wild-type form (CM-1 [Fig. 4], PAb1801, DO7); Pab240 was immunolocalised in those cases in a few nuclei only. In addition, 9 cases contained scattered *p53*-positive nuclei (less than 1%). All other cases lacked p53 immunostaining completely. Five to ten per cent positive tumour cell nuclei were found in three cases with uneventful clinical outcome and in two cases with local recurrence (Chi-square-test: not significant); the case with the development of lymph node metastases exhibited less than 5% positive nuclei. The immunohistochemical results were not significantly associated with tumour location and size.

SSCP analysis of exons 4–8 revealed no significant alterations in the mobility shift of the single-strand DNAs in all 10 cases investigated (Fig. 5).

Discussion

Our results from immunohistochemistry and SSCP analysis indicate that *p53* gene mutations probably have only a minor role in the genesis of Merkel cell carcinoma of the skin. To our knowledge, this is the first report on Merkel cell carcinoma dealing with *p53* analysis at both the protein and the genetic level. With application of four different anti-p53 antibodies (against different epitopes of the *p53* gene product) after wet autoclave pretreatment for antigen retrieval [2], only 5 of the 25 Merkel cell carcinomas in the present series (20%) showed between 5% and 10% positive tumour cell nuclei; an additional 9 cases (38%) contained only a few scattered positive nuclei. However, these results were not considered as typical *p53* overexpression suggestive of p53 gene mutations. The subsequently performed SSCP analyses of exons 4–8 suggest that the immunohistochemically observed p53 was not due to a mutation of the *p53* gene, although we cannot completely, exclude this owing to the small number of cells showing immunohistochemical p53 accumulation; it is possible that *p53* mutations were missed by SSCP analysis. However, it is unlikely that our SSCP approach failed to indicate genetic *p53* alterations within five exons of all five cases in which the immunohistochemically demonstrable p53 protein accumulation was investigated.

Immunohistochemistry for p53 protein expression has been suggested as a valid screening method for predicting underlying mutations in the *p53* gene in a variety of human malignancies [4, 9, 31]. However, several recent reports have described p53 protein accumulation independently of genetic alterations within a wide range of malignant tumours [7, 14, 33] and in normal somatic cells of members of a cancer family [3]. These results indicate that detectable amounts of p53 protein in cells may reflect stabilisation of the protein via interactions with other intracellular proteins (e.g. SV40 large T antigen [18], adenovirus E1B protein [32], HPV16 and HPV18 E6 oncoprotein [38], members of the heat shock protein family [19] mdm-2 oncoprotein [26, 29, 33] or transcriptional induction [36] rather than intragenetic mutations of the *p53* locus. Since all tumours were localised in sun-exposed areas of the skin, the possibility of reactive p53 expression caused by UV light exposure [6, 30] cannot be ruled out. However, the low number of nuclei positively stained for p53 in Merkel cell carcinoma (at the most 10%) indicates that inactivation of the p53 protein by one or more of the mechanisms mentioned above may also play only a minor part role in tumour progression in the cas of Merkel cell carcinoma.

Morphological and immunohistochemical findings have suggested two different types of Merkel cell carcinoma [5, 13, 15]. Since no correlation was observed between *p53* expression and the various antibodies investigated (neuroendocrine markers, cytoskeleton proteins, epithelial markers), the results of the present study has shed no further light on these suggestions.

Although the exact origin of Merkel cell carcinoma of the skin is not established [5, 11, 23, 34, 39], there is no doubt about its neuroendocrine nature. p53 mutations seem to be very rare events in other neuroendocrine tumours, including medullary thyroid carcinoma and pheochromocytoma [40], gastrointestinal and pancreatic carcinoids [23], and bronchial carcinoids [21]. Our findings thus support the concept that *p53* gene mutations are relatively unimportant in the genesis and progression of neuroendocrine tumours [37], with the possible exception of small cell carcinomas of the lung [22]. Although we cannot completely rule out the occurrence of *p53* mutations (nonsense or missense mutations) not leading to accumulation of the gene product in those cases not investigated by SSCP analysis, *p53* apparently contributes to neither the initiation nor the progression of Merkel cell carcinoma of the skin.

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