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Mutations of the PH Domain of Protein Kinase B (PKB/AKT) Are Absent in Human Epidermal Skin Tumors

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Key Words
Tumor suppressor gene · Tumorigenesis · Point mutations

Abstract

Background: While for most human solid tumors genetic alterations of few distinct genetic regions have been found, studies on basal cell carcinomas (BCC) have shown the prevalence of several abnormalities including alterations of the three *ras* genes, GAP (GTPase activating protein), p53, PTCH (the human homologue of Drosophila patched) and SMOH (the human homologue of Drosophila smoothened). On the other hand, during the last decade, a new oncogene, protein kinase B (PKB/AKT), has been characterized and found to be overexpressed in certain human tumors. In vivo activation of PKB/AKT necessitates its recruitment to the cell membrane mediated by the N-terminal pleckstrin homology (PH) domain. ***Objective:*** We investigated whether mutations of this mandatory domain are present in a subset of human epidermal skin tumors. ***Methods:*** RNA of 19 human skin tumors including 13 BCC, 4 squamous cell carcinomas (SCC; including 1 keratoacanthoma) and 2 neurofibromas of different size and tumor stage were used for reverse transcription and subsequent PCR amplification of the PH domain of PKB/AKT. ***Results:*** Cycle sequencing of the purified PCR products did not reveal any mutation of the PH domain of PKB/AKT. ***Conclusion:*** In human BCC and SCC, mutations of the PH domain of PKT/AKT do not play a major role during the carcinogenesis of these tumors.

Introduction

Epidermal skin cancer is the most common form of human cancer and basal cell carcinoma (BCC) is the most common form of epidermal skin cancer. In the search for the molecular basis of BCC, several known oncogenes and tumor suppressor genes have been analysed and alterations of *ras*, GAP, p53 have been found [for reviews see ref. 1, 2]. Since the isolation of the human PTCH gene [3], mutations in components of the hedgehog signaling pathway have been identified not only in nevoid basal cell carcinoma syndrome (NBCCS) but also in various, but not all, sporadic BCC [for reviews, see ref. 1, 4].

Comparison of allelic loss in BCC and squamous cell carcinoma (SCC) revealed a different general pattern of allelic deletion on chromosome 9 [5]. Analysis of PTCH revealed not only the absence of mutations but also normal expression of PTCH in SCC suggesting no involvement of the hedgehog signaling pathway in SCC [6, 7] and thus a different genetic basis for the neoplastic development of BCC and SCC.

In the early nineties, two groups cloned a new gene with high sequence homology to both families of serine/

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Table 1. Patient and tumor parameters used for the mutation analysis of the PH domain of PKB/AKT in human skin tumors

Tumor	Sex	Age	Histology	Localization	PH domain
1	m	56	BCC	nose	–
2	f	86	BCC	occipital	–
3	m	87	BCC	occipital	–
4	m	85	BCC	back	–
5	m	71	BCC	nose	–
6	f	79	BCC	leg	–
7	f	36	BCC	abdominal	–
8	f	85	BCC	parietal	–
9	f	60	BCC	back	–
10	m	84	BCC	nose	–
11	m	50	BCC	ear	–
12	m	80	BCC	neck	–
13	m	50	BCC	back	–
14	m	66	SCC	lip	–
15	m	84	Bowen	nose	–
16	f	60	ke.ac.	leg	–
17	m	74	Bowen	parietal	–
18	f	55	NF	back	–
19	f	37	NF	back	–

ke.ac. = Keratoacanthoma; NF = neurofibroma.

threonine kinases, protein kinase A and C. A third group demonstrated a viral oncogene, v-*akt* to be homologous to the new gene, named protein kinase B or AKT. A third synonym, RAC protein kinase, is no longer used to avoid confusion with rac GTPase [for review, see ref. 8].

Growth-factor-induced PKB/AKT activation is mediated by phosphatidylinositol-3 kinase (PI3K) which in turn activates PDK 1 and 2 [for a review, see ref. 9] by the production of phosphatidylinositol(3,4,5)triphosphate lipids. The lipids also recruit PKB/AKT to the plasma membrane by means of its PH domain [10], and PKB/AKT is subsequently phosphorylated, resulting in full activation [8, 11]. Several lines of evidence suggest that components of this pathway are involved in oncogenic transformation. First, PI3K exists in an oncogenic form [12]. In cells that harbor this oncogene, high PKB/AKT activity is observed [13]. Second, PKB/AKT fused to the membrane-targeting *gag* sequence is able to transform fibroblasts [14], indicating that for full activation of PKB/AKT the PH domain, by which physiological membrane recruitment takes place, is mandatory [for a review, see ref. 10]. Third, a negative regulator of PKB/AKT, PTEN [15], has been described and characterized as tumor suppressor gene. Deletion or mutation of PTEN has been found in tumor cells and correlates with high amounts of PKB/AKT activity [16–20] in these transformed cells. Taken together, these findings argued in favor of the possibility that PKB/AKT might be activated in human tumors. An altered membrane affinity of PKB/AKT, mediated through its PH domain, should be able to activate PKB/AKT constitutively despite the fact that no gain-of-function mutation of this domain is known to date.

We therefore examined a panel of 19 human epidermal skin tumors of the prevalence of mutations in the PH domain of PKB/AKT and due to its different molecular biology [5–7] included both BCC and SCC in this study. Additionally we analyzed 2 neurofibromas (table 1).

Material and Methods

Subjects and Skin Tumors

Tissue of 19 patients (11 males and 8 females of Caucasian origin aged between 28 an 87 years) who underwent surgery for histologically proven epidermal skin tumors at the Dermatological Department of the University of Heidelberg, Germany, was obtained directly at the time of the excision. The excised specimens were divided at surgery, a small part of the tumor tissue adjacent to the thickest tumor portion was used for the biochemical analysis. The remaining parts of the tumors were fixed in formalin and embedded in paraffin for histological analysis. The tumors were 13 BCC, 4 SCC and 2 neurofibromas. The patients had not received prior therapy.

Synthetic Oligonucleotides

According to the sequence published by Jones et al. [21], gene bank accession No. M 63167, two primers up- and downstream of the PH domain of PKB/AKT were designed and synthesized by Eurogentec, Cologne, Germany. Upon PCR (see below) both primers (5′-GGAGCCTCGGGCACCATGAGC-3′; 5′-CCGGAAGTCCATCTCCTCCTC-3′) resulted in an amplification product of 378 bp.

RT-PCR for PKB/AKT

For RT, between 100 and 400 ng of total tumor RNA, prepared according to a modified GTC method using selective binding properties of a silica gel described by Marquardt et al. [22] (Qiagen, Hilden, Germany), were added to 20 µl of RT buffer containing 10 m*M* DTT, 0.5 m*M* dNTPs, 500 ng oligo(dT)$_{12–18}$ primer, 200 units superscript II reverse transcriptase (Gibco) according to the manufacturer's instructions and incubated for 60 min at 42 °C.

PCR was carried out in 50-µl samples containing 100 ng cDNA, 2.5 units AmpliTaqGold (Perkin Elmer), 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 0.4 µ*M* oligonucleotides in 1 × PCR buffer (Perkin Elmer) in a thermal cycler for 30 s at 95 °C, 30 s at 56 °C, 60 s at 72 °C for 45 cycles.

The PCR amplification product comprised a sequence of 378 bp in length. Amplification products were controlled by agarose gel electrophoresis for purity and expected length (fig. 1). The specificity of amplification was checked by negative control samples containing complete amplification medium but no tissue extract. The integrity of the cDNA preparation was confirmed by RT-PCR of β2-microglobulin in each sample, resulting in an amplification product of 136 bp (data not shown).

DNA Sequencing

PCR products were purified by electrophoresis and subsequent column elution (Qiagen) and sequenced by use of each of the amplification primers by fluorescence cycle sequencing (Seqlab, Göttingen, Germany).

Sequence Analysis

Sequence analysis was performed at the National Center for Biotechnology Information using the Basic Local Alignment Search Tool (www.ncbi.alm.nih.gov/BLAST/).

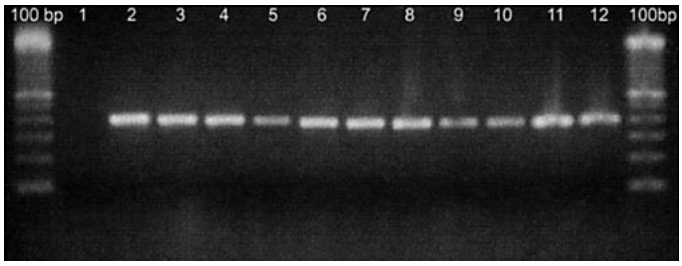

Fig. 1. RT-PCR analysis of the PH domain of PKB/AKT RNA prepared from specimens from 11 patients (lanes 2–12). A length standard is indicated (100 bp) and a negative control (lane 1) is included. An amplification product of 378 bp is visible in all 11 probes.

Results

We examined human epidermal skin tumor specimens of 19 patients. Of these 19 skin tumors, 13 were BCC of different size and localization. We also included 2 neurofibromas, 1 keratoacanthoma, 2 Bowen's carcinomas and 1 SCC. Despite the fact that intact tumor RNA had to be prepared, we succeeded in amplifying the critical part of PKB/AKT by our RT-PCR protocol. Using appropriate primers located at the PH domain boundaries, the expected 378-bp PCR product was obtained (fig. 1). After the 378-bp fragment was purified, DNA sequencing was performed. In no single case did sequencing of the PH domain disclose a mutation. An example is given in figure 2.

Discussion

Several genetic alterations have been found in human epidermal skin carcinomas, including alterations of *ras*, p53, GAP, PTCH and SMOH [1, 2]. Two genes have been extensively investigated in skin cancers, p53 and *ras*: point mutations of *ras* genes have been detected in both, SCC and BCC, albeit at a rather low prevalence [e.g. 23, 24]. The predominant codon 12-point mutation might be explained by a UV-induced DNA damage and thus can serve as an explanation for the clinical observation that UV exposure is an important risk factor for the development of epidermal skin cancer [24, 25]. This is even more apparent for the kind of mutation found in the p53 tumor suppressor gene of epithelial skin tumors: the majority of mutations were found at bipyrimidines, where a transition mutation from cytosine to thymidine occurred and is at the same time a typical marker lesion for UV damage of the DNA [26]. Despite the obvious association between sunlight and the incidence of epidermal skin cancer [27–30], the prevalence of these pathognomonic mutations in known cancer-related genes is rather low and this suggests that further oncogenic proteins may be involved in signal transduction from the extracellular environment, through the cell membrane, into the cytoplasm towards the nucleus, where transcription is modified and thus contribute to the tumor phenotype [31]. In 1996, mutations of newly discovered tumor, suppressor gene, the human homologue of Drosophila patched, PTCH, were found to be responsible for NBCCS [3, 32]. The described mutations were UV-independent in hereditary BCC, but were possibly induced by UV in sporadic BCC [3, 32]. The analysis of PTCH in SCC revealed the absence of mutations, and also a normal expression of PTCH, which suggested no involvement of components of the hedgehog signaling pathway in SCC [6, 7]. Independently of this, in the context of a presumed multistep carcinogenesis of at least certain epidermal skin cancers, this tumor type seems to be especially interesting because its behavior ranges from extremely indolent to very aggressive [33].

In the present study, human epidermal skin tumors have been tested for the first time for mutations in PKB/AKT. Nineteen skin tumors of different histologic characterization were included. Mutations of the PH domain of PKB/AKT were detected neither in SCC nor in BCC. Thus, the examined hypothesis that PKB/AKT might be mutated in human epidermal skin carcinogenesis could not be confirmed. The reasons for the complete absence of PKB/AKT mutations remain speculative.

First, it might well be that mutations of the PKB/AKT oncogene are not involved in human skin tumors. This is known from other tumor types that lack frequent oncogene activation, e.g. human breast cancer cells very rarely contain activated *ras* genes, whereas most epithelial tumors do; breast tumors from various other species exhibit these mutations as well [for a review, see ref. 2]. In these tumor types, alterations of signal transduction pathways occur at other levels (e.g. BRCA). This first hypothesis

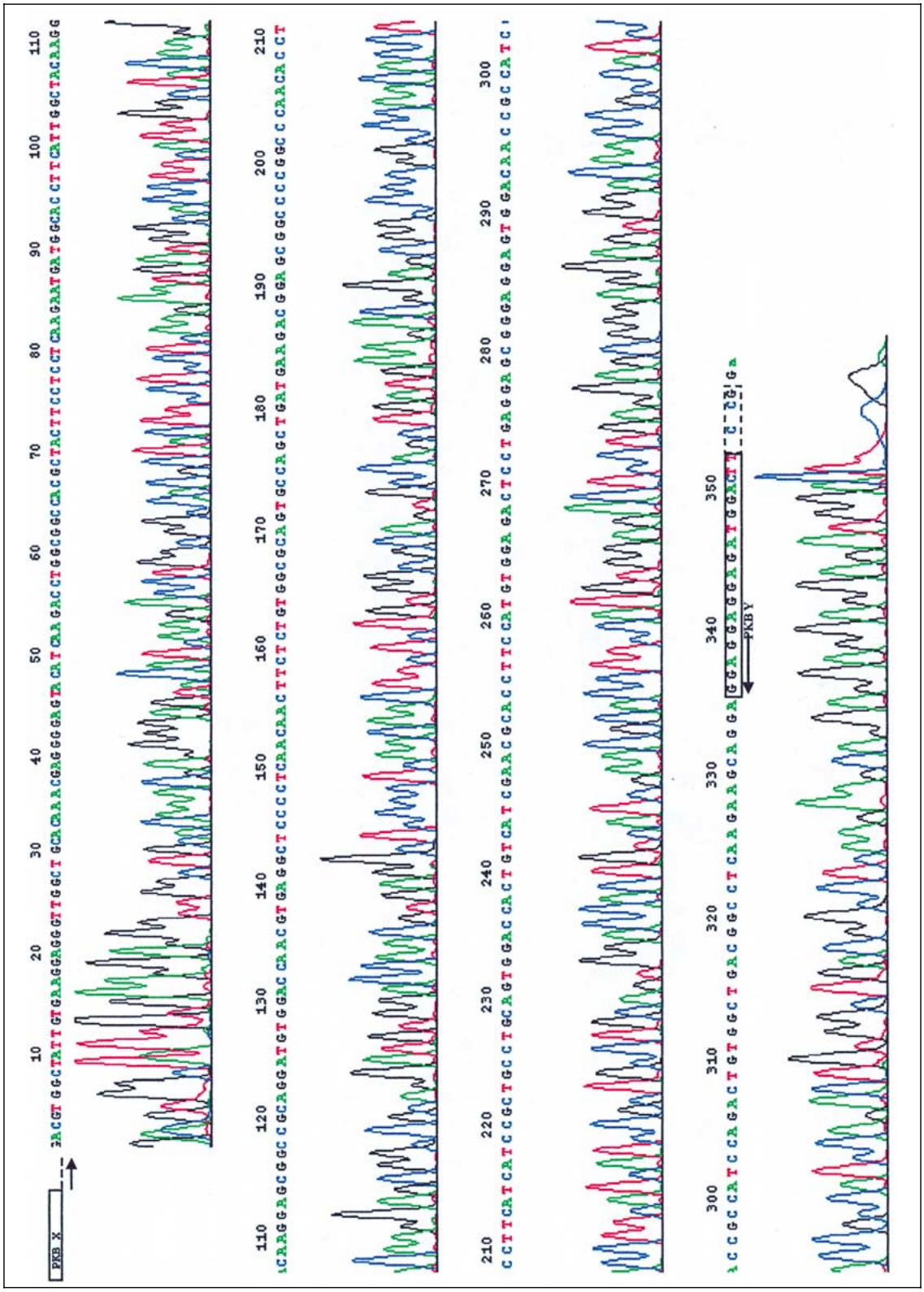

Fig. 2. Cycle sequencing of the PH domain of PKB/AKT from a purified PCR product (fig. 1). The sense sequence is shown and the position of both primers is indicated. Comparison of the shown sequence with the result of the opposite strand and the known wild-type sequence did not reveal any mutation of the PH domain.

might be confirmed by analysis of other types of tumors, for example further skin tumor entities. Melanomas do not harbor mutations of the PH domain [34].

Second, PKB/AKT might be a signal transduction component with in vitro oncogenic potency that is not found mutated in tumors. An example for this is raf-1, an effector of *ras*, which has not been found mutated in human tumors [35]. Interestingly, raf-1 also is a serine/threonine kinase located in its active form at the inner cell membrane. This hypothesis, too, might be examined by studies on PKB/AKT activation in other tumor types, also of other organ systems.

References

1 Booth D: The hedgehog signalling pathway and its role in basal cell carcinoma. Cancer Metastasis Rev 1999;18:261–284.
2 Waldmann V, Rabes H: What's new in ras genes? Physiological role in significant transduction and significance of ras gene activation in tumorigenesis. Pathol Res Pract 1996;192: 883–891.
3 Johnson R, Rothman A, Xie J, Goodrich L, Bare J, Bonifas J, Quinn A, Myers R, Cox D, Epstein E, Scott M: Human homologue of patched, a candidate gene for basal cell nevus syndrome. Science 1996;272:1668–1671.
4 Sidransky D: Is human patched the gatekeeper of common skin cancers? Nat Genet 1996;14: 7–8.
5 Quinn A, Sikkink S, Rees J: Basal cell carcinomas and squamous cell carcinomas of human skin show distinct patterns of chromosome loss. Cancer Res 1994;54:4756–4759.
6 Eklund L, Lindstrom E, Unden A, Lundh-Rozzell B, Stahle-Backdahl M, Zaphiropoulos P, Toftgard R, Soderkvist P: Mutation analysis of the human homologue of Drosophila patched and the xeroderma pigmentosum complementation group A genes in squamous cell carcinomas of the skin. Mol Carcinog 1998;21:87–92.
7 Petrianu A, Boson W, Bale A, Friedman E, DeMarco L: Mutational analysis of candidate genes in human squamous cell carcinomas. Laryngoscope 1999;109:661–663.
8 Kops G, Burgering B: Forkhead transcription factors: New insights into protein kinase B (c-akt) signaling. J Mol Med 1999;77:656–665.
9 Vanhaesebroeck B, Alessi D: The PI3K-PDK1 connection: More than just a road to PKB. Biochem J 2000;346:561–576.
10 Lemmon M, Ferguson K: Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem J 2000;350:1–18.
11 Chan T, Rittenhouse S, Tsichlis P: AKT/PKB and other D3 phosphoinositide-regulated kinases: Kinase activation by phosphoinositide-dependent phosphorylation. Annu Rev Biochem 1999;68:965–1014.
12 Jimenez C, Jones D, Rodriguez-Viciana P, Gonzalez-Garcia A, Leonardo E, Wennstrom S, von Kobbe C, Toran J, Borlado L, Calvo V, Copin S, Albar J, Gaspar M, Diez E, Marcos M, Downward J, Martinez A, Merida I, Carrera A: Identification and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase. EMBO J 1998; 17:743–753.
13 Chang H, Aoki M, Fruman D, Auger K, Bellacosa A, Tsichlis P, Cantley L, Roberts T, Vogt P: Transformation of chicken cells by the gene encoding the catalytic subunit of PI3K. Science 1997;276:1848–1850.
14 Bellacosa A, Testa J, Staal S, Tsichlis P: A retroviral oncogene, akt, encoding a serine-threonine kinase containing a SH2-like region. Science 1991;254:274–277.
15 Vazquez F, Sellers W: The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signaling. Biochim Biophys Acta 2000;1470:M21–M35.
16 Haas-Kogan D, Shalev N, Wong M, Mills G, Yount G, Stokoe D: Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor TPEN/MMAC. Curr Biol 1998;8:1195–1198.
17 Wu X, Senechal K, Neshat M, Whang Y, Sawyers C: The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. Proc Natl Acad Sci USA 1998;95:15587–15591.
18 Davies M, Lu Y, Sano T, Fang X, Tang P, LaPushin R, Koul D, Bookstein R, Stokoe D, Yung W, Mills G, Steck P: Adenoviral transgene expression of MMAC/PTEN in human glioma cells inhibits AKT activations and induces anoikis. Cancer Res 1998;58:5285–5290.
19 Birck A, Ahrenkiel V, Zeuthen J, et al: Mutation and allelic loss of the PTEN/MMAC1 gene in primary and metastatic melanoma biopsies. J Invest Dermatol 2000;114:277–280.
20 Reifenberger J, Wolter M, Bostrom J, et al: Allelic losses on chromosome arm 10q and mutation of PTEN(MMAC1) tumor suppressor gene in primary and metastatic malignant melanomas. Virchows Arch 2000;436:487–493.
21 Jones P, Jakubowicz T, Pitossi F, Maurer F, Hemmings B: Molecular cloning and identification of a serine/threonine protein kinase of the second messenger subfamily. Proc Natl Acad Sci USA 1991;88:4171–4175.
22 Marquardt O, Ahl R, Haas B, Ohlinger V, Straub O: Methods for a rapid extraction of RNA of clinical samples for use in diagnostic RT-PCR. Med Microbiol Lett 1996;5:55–75.
23 Pierceall W, Goldberg L, Tainsky M, Mukhopadhyay T, Ananthaswamy H: Ras gene mutation and amplification in human nonmelanoma skin cancers. Mol Carcinog 1991;4:196–202.
24 Spencer J, Kahn S, Jiang W, DeLeo V, Weinstein I: Activated ras genes occur in human actinic keratoses, premalignant precursors to squamous cell carcinomas. Arch Dermatol 1995;131:796–800.
25 Veer L van't, Burgering B, Versteeg R, Boot A, Ruiter D, Osanto S, Schrier P, Bos J: N-ras mutations in human cutaneous melanoma from sun exposed body sites. Mol Cell Biol 1989;9:3113–3116.
26 Greenblatt N, Bennett W, Hollstein M, Harris C: Mutations in the p53 tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. Cancer Res 1994;54:4855–4878.
27 English D, Armstrong B, Kricker A, Fleming C: Sunlight and cancer. Cancer Causes Control 1997;8:271–283.
28 Quinn A: UV radiation and skin carcinogenesis. Br J Hosp Med 1997;58:261–264.
29 Gailani M, Leffell D, Ziegler A, Gross E, Brash D, Bale A: Relationship between sunlight exposure and a key genetic alteration in basal cell carcinoma. J Natl Cancer Inst 1996;88:349–354.
30 Waldmann V, Bock M, Jäckel A, Deichmann M, Dockendorff K, Näher H: Molecular biology of malignant melanoma. Hautarzt 1999;50: 398–405.
31 Tsatsanis C, Spandidos D: The role of oncogenic kinases in human cancer. Int J Mol Med 2000;5:593–590.
32 Hahn H, Wicking C, Zaphiropoulos P, Gailani M, Shanley S, Chidambram A, Vorechowsky I, Homberg E, Unden A, Gillies S, Negus K, Smyth I, Pressman C, Leffell D, Gerrard B, Goldstein A, Dean M, Toftgard R, Chenevix-Trench G, Wainwright B, Bale A: Mutations of the human homolog of Drosophila patched in the naevoid basal cell carcinoma syndrome. Cell 1996;85:841–851.
33 Jacobs G, Rippey J, Altini M: Pepdiction of aggressive behavior in basal cell carcinoma. Cancer 1982;49:533–537.
34 Waldmann V, Wacker J, Deichmann M: Absence of mutations in the PH domain of protein kinase B (PKB/AKT) in malignant melanoma. Melanoma Res, in press.
35 Naumann U, Eisenmann-Tappe I, Rapp U: The role of Raf kinases in development and growth of tumors. Rec Results Cancer Res 1997;143:237–244.