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RESEARCH

B-Raf Mutations, Microsatellite Instability and p53 Protein Expression in Sporadic Basal Cell Carcinomas

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Abstract Basal Cell Carcinoma (BCC) is the most common skin malignancy. Genes related to the Ras/Raf signalling pathway have been implicated in the pathogenesis of skin cancer. The objective of this study was to investigate the presence of B-Raf mutations in sporadic BCCs as well as its correlation with the phenotype of microsatellite instability (MSI), the clinicopathological parameters of the tumours and p53 protein expression. 83 BCC specimens were screened for B-Raf mutations, applying polymerase chain reaction, single-stranded conformation polymorphism (PCR-SSCP) and DNA sequencing. MSI status was examined using mononucleotide microsatellite markers and p53 protein expression was demonstrated by immunohistochemical staining. A C to T transition at 1790 nucleotide leading to a silent mutation L597L; and a T to A transversion causing an amino acid change (F610I) have been found. MSI was detected in 5% of the cases and p53 accumulation was present in 37/83 samples studied. Although rare B-Raf alterations have been observed in BCC, none of them harboured the hot-spot mutation T1799A commonly present in melanomas and colon carcinomas. Consequently, no correlation could be determined between B-Raf alterations, MSI status, the clinicopathological features and p53 protein expression. Our results are in favour of a secondary importance for Ras signalling cascade genes in BCC pathogenesis.

Keywords Basal cell carcinoma · B-Raf · Microsatellite instability · p53 · Skin

Abbreviations

BCC	Basal cell carcinoma
MSI	Microsatellite instability
MSS	microsatellite stable
MMR	Mismatch repair
PCR	Polymerase chain reaction
SSCP	single-stranded conformation polymorphism
PTCH	patched
SMOH	smoothened
H-MSI	high-level microsatellite instability
MAPK	mitogen-activated protein kinase
ERK	extracellular signal regulated kinase
MEK	MAPK/ERK kinase
SCC	squamous cell carcinoma

Introduction

Basal Cell Carcinoma (BCC) is the most common cutaneous cancer. It is considered as a malignancy of benign clinical course [1]. BCCs are characterized by slow progression and a low frequency of metastasis. A subgroup of BCCs—metatypical, infiltrative and schlerosing subtypes—demonstrate an histologically aggressive growth pattern [2], whereas other subtypes such as nodular or superficial constitute an indolent-growth subgroup [2]. Keratotic, infudibulocystic, follicular, fibroepithelioma of Pinkus are variants of BCC histology spectrum showing a

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range of specific cell lineage differentiation, not impacting prognosis however [2].

Alterations affecting several cancer related genes such as those of the Sonic Hedgehog pathway, patched (PTCH) and smoothened (SMOH), the p53 tumour suppressor gene as well as genes related to the Ras/Raf signalling pathway have raised increasing interest for the pathogenesis of skin cancer and have been suggested as potential therapeutic targets [3–5].

Numerous cellular responses are regulated by the Ras-Raf-MEK-ERK kinase cascade. Ras is activated by the extracellular binding of growth factors to tyrosine kinases and subsequently activates the Raf family of serine/threonine protein kinases. Raf phosphorylates the mitogen-activated protein kinase extracellular signal regulated kinase (MAPK/ERK kinase, MEK) which then stimulates the extracellular signal regulated kinase (ERK), which controls many cytoplasmic and nuclear proteins [6]. The Raf family consists of A-Raf, B-Raf and C-Raf protein kinases which play distinct roles in procedures such as apoptosis, differentiation and proliferation [7]. Activating B-Raf mutations occur frequently in cutaneous malignant melanomas and melanoma cell lines [7, 8]. They have also been associated with mismatch repair (MMR) deficient colorectal cancers [9, 10]. The most common B-Raf mutation, affecting exon 15, is a transversion of thymine to adenine at nucleotide 1799 resulting in a substitution of valine by glutamic acid at codon 600 [7–10]. C-Raf mutations are rarely encountered whereas A-Raf mutations have not yet been identified. It could be hypothesized that although B-Raf is activated by just one amino acid substitutions, two mutations could be necessary for A-Raf or C-Raf oncogenic stimulation [6].

The objective of the present study was to evaluate the mutational status of B-Raf and its possible importance for the pathogenesis of sporadic BCCs in relation to their MSI status, their clinicopathological parameters and p53 protein expression.

Material and Methods

Tumour Specimens Eighty three tumour specimens from Greek patients diagnosed with BCC, were available for examination. The proportion of neoplastic cells was more than 75% in each block, as estimated by histopathological analysis. BCCS were subdivided as metatypical(n=8), schlerosing(n=2) and infiltrative(n=9)—histologically aggressive growth—and nodular(n=36), follicular(n=6), superficial(n=13), adenocystic(n=3), fibroepithilioma of Pinkus(n=3), keratotic(n=2), signet ring(n=1) by examination of haematoxylin/eosin stained sections. Perineural invasion was present in eight samples. In twenty-seven cases matched normal tissue was available for molecular analysis.

Genomic DNA Isolation DNA extraction was performed by standard protocols of Proteinase K digestion followed by phenol/chloroform extraction as previously described [11].

MSI Assessment Microsatellite instability assessment was performed using the monomorphic mononucleotide marker BAT26 which is highly sensitive for MSI+ [12, 13].

B-RAF Exon 15 Analysis Genomic DNA (300 ng) was amplified in a 25 μl reaction mixture. PCR was performed in a Progene Techne thermal cycler. Amplification consisted of an initial step of 2 min at 94°C, followed by 40 cycles of denaturation at 94°C, annealing at 60°C, extension at 72°C followed by a 7 min final extension. Taq. DNA polymerase (Titanium, BD Biosciences, USA) was used. The primer sequences used for the amplifying of the fragment encompassing the exon 15 of B-Raf gene were: B-Raf exon 15 (forward) 5′-CATAATGCTTGCTCTGATAGG-3′; B-Raf exon 15 (reverse) 5′-GGCCAAAAATTTAATCAGTGGA-3′.

PCR products for exon 15 of the B-RAF gene were examined for mutations using a 0.5X MDE gel (Cambrex Bio Science, Rockland Inc. Rockland ME USA) electrophorized at 4°C overnight, visualized by silver staining and examined for abnormal band patterns. Human cancer cell line HT29, determined as mutated in exon 15 of the B-RAF gene, was used as a positive control. *Sequencing:* PCR products for exon 15 of the B-RAF gene with an abnormal SSCP band pattern were sequenced using the Big Dye terminator cycle sequencing kit (Applied Biosystems, California, USA). The PCR primers were used for sequencing reactions as well. The sequencing products were analyzed on an ABI prism 310 Genetic Analyzer (Perkin-Elmer, California, USA).

Immunohistochemistry p53 immunostaining was performed in formalin-fixed paraffin-embedded material using the monoclonal anti-p53 antibody DO-7 (DAKO, Glostrup, Denmark) which identifies both the wild and the mutant forms of p53 protein. Immunohistochemistry assessment was performed as previously described [11]. Immunostaining was evaluated after light microscope evaluation in at least 10 high power fields throughout the tumour area. The immunopositive status for p53 marker was defined as the presence of more than 10% stained tumour cells exhibiting nuclear staining with moderate to strong intensity among all tumour cells examined. Cases with less than 10%, for p53 protein, stained tumour cells or cases with weakly stained tumour cells were determined as immunonegative.

Statistical Analysis Data were analysed by x^2 test, considering the presence or absence of B-Raf mutations and the presence of MSI; the clinicopathological features (namely age, sex, presence of perineural invasion and anatomical site).

Results

Eighty three (83) tumour samples from patients with basal cell carcinoma were screened for point mutations in exon 15 of the B-Raf gene using PCR-SSCP analysis. Direct sequencing was conducted in samples showing abnormal bands.

B-Raf mutations were detected in two out of eighty three BCCs. In the first case, a fibroepithelioma of Pinkus from a male patient, located on the face, a C to T substitution at nucleotide 1790 was observed, leading however to a silent mutation (L597L). This case exhibited alteration of the very sensitive mononucleotide marker BAT26 and was thus considered as displaying high-level microsatellite instability (H-MSI). BAT26 has been recommended to be used as a single marker for MSI screening and classification due to it's highly sensitivity to MSI with no necessity to study normal tissue [12, 13]. This case did not express p53 protein.

In the second case, a nodular BCC, characterised histologically as a non-aggressive subtype, located on the face of a female patient aged 65 year-old, a T to A substitution at nucleotide 1829 leading to an exchange from phenylalanine to isoleucine (F610I) was observed (Fig 1). This case displayed no alteration of the microsatellite marker BAT-26 and so could be considered as microsatellite stable (MSS). p53 protein expression was negative also for this case.

The common V600E B-Raf point mutation was not detected in any case.

MSI was detected in 5% of the cases in our cohort. Analysis of p53 status as assessed by immuhistochemistry resulted positive in 37/83 samples (45%) for p53 protein immunoexpression.

No significant associations between B-Raf mutations and the presence of MSI, p53 expression and/or the clinicopathological features (age, sex, presence of perineural invasion and anatomical site) could be determined.

Discussion

Recently, novel therapeutic approaches such as targeted therapies focusing on the inhibition of Ras/Raf signalling cascade have been suggested as promising options for cancer treatment [14]. Green et al (2004) [3] proposed that inhibitors for the abovementioned pathway- among others- may present potential agents for molecular therapy in melanoma and non-melanoma skin cancer.

We have analysed eighty three BCCs for mutations in the activation segment of the kinase domain of B-Raf gene (exon 15) and examined their possible association with the presence of MSI, p53 expression and standard clinicopathological parameters. The screening for mutations affecting only exon 15 of B-Raf appears to be sufficient since mutations are distributed mostly in exons 11 and 15 of the gene and about 80% of them are a T→A transversion at nucleotide 1799, leading to a substitution of glutamic acid for valine at codon 600 (formerly known as V599E) in exon 15 [8, 15]. In our cohort absence of the above alteration was observed, indicating that this hot spot mutation may not be involved in the tumorigenesis of BCCs. Our results are in accordance to those published by other authors [4, 16], supporting that B-Raf gene does not appear to carry activated mutations in non-melanoma skin tumours such as BCC.

However, in two cases we found single base substitutions in B-Raf exon 15. For the first case, a C to T transition at nucleotide 1790 was observed, leading to a silent mutation (L597L). Interestingly, BAT26 microsatelllite marker was also altered in this case displaying lack of hMLH1 protein immunoexpression. The reason of hMLH1 inactivation could be attributed to promoter hypermethylation as indicated by the absence of immunohistochemical expression of the protein. A strong correlation between B-Raf mutations (V600E point mutation), MMR deficiency and hMLH1 hypermethylation has been suggested for colorectal cancers [10]. This specimen

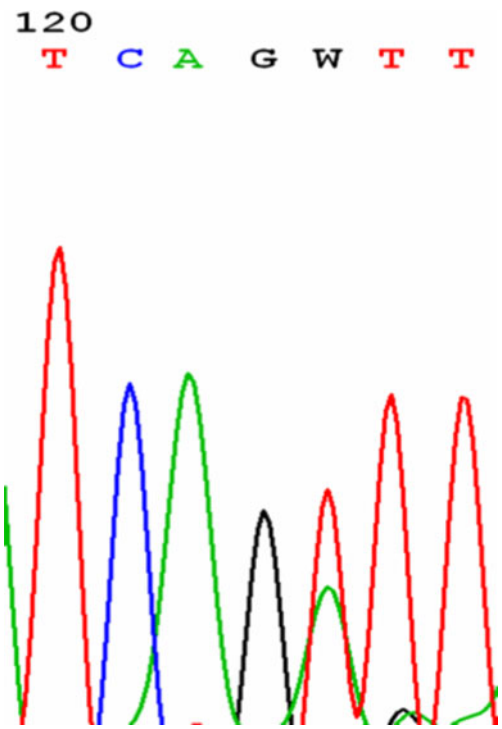

Fig. 1 The T-to-A tranversion at nucleotide1829 in exon 15 of the B-Raf gene, that leads to an exchange from phenylalanine to isoleucine at codon 610 (F610I)

could represent such a case showing MSI instability, hMLH1 inactivation as well as an alteration in B-Raf gene, although of unknown impact for the activity of the protein.

In the second case, we detected a T to A transversion leading to an exchange from phenylalanine to isoleucine (F610I). This case was immunonegative for p53 protein, suggesting a normal p53 gene function.

We found p53 expression in a percentage of 45% (37/83) of the BCCs studied, which seems to be at the lower end among different reports of p53 expression levels in BCCs. In those studies over-expression of p53 ranges from 40 to 90% [17–26]. The above variations could be attributed to the use of different antibodies and/or staining procedures applied for the detection of p53 protein expression as well as to differences in the assessment of the results. Furthermore, ethnical differences could also influence the outcome.

To our knowledge this appears to be the first study investigating the presence of MSI in parallel with B-Raf mutations in BCCs. MSI was detected only in a small proportion (5%) of our samples hence a correlation between B-Raf mutations and MSI status in BCCs could not be substantiated. Other studies have reported a low frequency of MSI in BCCs. Quinn et al. detected MSI in a percentage lower than 5% [27] whereas a recent study reported that BCCs of azathioprine-treated organ transplant recipients were microsatellite stable even in the highly MSI-sensitive BAT25 and BAT26 markers [28]. Moreover Young et al reported no MSI in 32 Squamous Cell Carcinomas (SCCs) after analyzing the sensitive BAT26 marker [29]. They also reported increased levels of MMR proteins (MSH2, MSH3, MSH6, MLH1 and PMS2) mainly in SCCs and at a lesser extent in BCCs compared with normal skin and no association between the presence of MSI and loss of expression of any examined MMR protein. Interestingly, they further describe a dysregulation of the MMR system [29]. The possible role of this dysregulation in the multistep process of carcinogenesis has to be elucidated.

Conclusively, we have examined a subset of 83 BCCs for B-Raf exon 15 activating mutations in parallel with their MSI status and p53 expression. The absence of B-Raf as well as MSI alterations at a considerable proportion of the analysed cases leads to the assumption that these could not be considered as causative events for the development of Basal Cell Carcinoma of the skin. The possible implication of the ERK signalling pathway in the pathogenesis of BCC mandates further investigation at the levels of MEK and ERK MAP kinases.

References

1. Ionescu DN, Arida M, Jukic DM (2006) Metastatic basal cell carcinoma: four case reports, review of literature, and immunohistochemical evaluation. Arch Pathol Lab Med 130:45–51
2. Crowson AN (2006) Basal cell carcinoma: biology, morphology and clinical implications. Mod Pathol 19(suppl 2):S127–S147
3. Green CL, Khavari PA (2004) Targets for molecular therapy of skin cancer. Semin Cancer Biol 14:63–69
4. Reifenberger J, Wolter M, Knobbe CB et al (2005) Somatic mutations in the PTCH, SMOH, SUFUH and TP53 genes in sporadic basal cell carcinomas. Br J Dermatol 152:43–51
5. Pons M, Quintanilla M (2006) Molecular biology of malignant melanoma and other cutaneous tumors. Clin Transl Oncol 8:466–74
6. Dhomen N, Marais R (2007) New insight into BRAF mutations in cancer. Curr Opin Genet Dev 17:31–39
7. Houben R, Becker JC, Kappel A et al (2004) Constitutive activation of the Ras-Raf signaling pathway in metastatic melanoma is associated with poor prognosis. J Carcinog 26:3–6
8. Davies H, Bignell GR, Cox C (2002) Mutations of the BRAF gene in human cancer. Nature 417:949–954
9. Rajagopalan H, Bardelli A, Lengauer C et al (2002) Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 418 (6901):934
10. Domingo E, Espín E, Armengol M et al (2004) Activated BRAF targets proximal colon tumors with mismatch repair deficiency and MLH1 inactivation. Genes Chromosom Cancer 39:138–142
11. Saetta AA, Aroni K, Stamatelli A et al (2005) Expression of mismatch repair enzymes, hMLH1 and hMSH2 is not associated with microsatellite instability and p53 protein accumulation in basal cell carcinoma. Arch Dermatol Res 297:99–107
12. Zhou XP, Hoang JM, Li YJ et al (1998) Determination of the replication error phenotype in human tumours without the requirement for matching normal DNA by analysis of mononucleotide repeat microsatellites. Genes Chromosom Cancer 21:101–107
13. de la Chapelle A (1999) Testing tumors for microsatellite instability. Eur J Hum Genet 7:407–408
14. Roberts PJ, Der CJ (2007) Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 26:3291–3310
15. Mercer KE, Pritchard CA (2003) Raf proteins and cancer: B-Raf is identified as a mutational target. Biochim Biophys Acta 1653:25–40
16. Libra M, Malaponte G, Bevelacqua V et al (2006) Absence of BRAF gene mutation in non-melanoma skin tumors. Cell Cycle 5:968–970
17. Ziegler A, Leffell DJ, Kunala S et al (1993) Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. Proc Natl Acad Sci USA 90:4216–4220
18. Shea CR, McNutt NS, Volkenandt M et al (1992) Overexpression of p53 protein in basal cell carcinomas of human skin. Am J Pathol 14:25–29
19. Barbareschi M, Girlando S, Cristofolini P et al (1992) p53 protein expression in basal cell carcinomas. Histopathology 21:579–581
20. De Rosa G, Staibano S, Barra E et al (1993) p53 protein in aggressive and non-aggressive basal cell carcinoma. J Cutan Pathol 20:429–434
21. Ro YS, Cooper PN, Lee JA et al (1993) p53 protein expression in benign and malignant skin tumours. Br J Dermatol 128:237–241
22. Barrett TL, Smith KJ, Hodge JJ et al (1997) Immunohistochemical nuclear staining for p53, PCNA, and Ki-67 in different histologic variants of basal cell carcinoma. J Am Acad Dermatol 37:430–437
23. Boonchai W, Walsh M, Cummings M et al (2000) Expression of p53 in arsenic related and sporadic basal cell carcinoma. Arch Dermatol 136:195–198
24. Demirkan NC, Colakoglu N, Düzcan E (2000) Value of p53 protein in biological behavior of basal cell carcinoma and in normal epithelia adjacent to carcinomas. Pathol Oncol Res 6:272–274
25. Auepemkiate S, Boonyaphiphat P, Thongsuksai P (2002) p53 expression related to the aggressive infiltrative histopathological feature of basal cell carcinoma. Histopathology 40:568–573

26. Ansarin H, Daliri M, Soltani-Arabshahi R (2006) Expression of p53 in aggressive and non-aggressive histologic variants of basal cell carcinoma. Eur J Dermatol 16:543–547
27. Quinn GA, Healy E, Rehman I et al (1995) Microsatellite instability in human non-melanoma and melanoma skin cancer. J Invest Dermatol 104:309–312
28. Wisgerhof HC, Hameetman L, Tensen CP et al (2009) Azathioprine-induced microsatellite instability is not observed in skin carcinomas of organ transplant recipients. J Invest Dermatol 129:1307–1309
29. Young LC, Listgarten J, Trotter MJ et al (2008) Evidence that dysregulated DNA mismatch repair characterizes human non-melanoma skin cancer. Br J Dermatol 158:59–69