High Frequency of Loss of Heterozygosity on Chromosome Region 9p21–p22 but Lack of $p16^{INK4a}/p19^{ARF}$ Mutations in Greek Patients with Basal Cell Carcinoma of the Skin

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Basal cell carcinoma of the skin is the most common neoplasia in humans. Previous studies have shown the existence of allelic imbalance (loss of heterozygosity and microsatellite instability) in BCC on several human chromosomes. Chromosome region 9p21–p22 harbors the *CDKN2a/p16*^{INK4a}, *p19*^{ARF}, and *p15*^{INK4b} tumor suppressor genes. To determine the contribution of these genes to the development of basal cell carcinomas we looked for evidence of allelic imbalance in 67 sporadic basal cell carcinoma specimens from Greek patients and screened 28 of them presenting loss of heterozygosity at 9p21–p22 for germline mutations in *p16*^{INK4a} and *p19*^{ARF} genes. Chromosome regions 17q21 and 17p13 were also screened for allelic imbalance in all the 67 basal cell carcinoma specimens. Overall, 69% (46 of 67) of the specimens displayed loss of heterozygosity in at least one microsatellite marker, whereas only six of the 67 (9%) exhibited microsatellite instability. For the 9p21–p22 locus the overall frequency of loss of heterozygosity reached 55% (37 of 67) and is the highest reported. The overall frequency of loss of heterozygosity for the 17q21 locus is 34% (22 of 64) and for the 17p13 locus is 11% (seven of 65). Two of the 28 loss of heterozygosity positive cases were heterozygous for a previously described polymorphism, Ala148Thr, in exon 2 of the *CDKN2a* gene. This is the first demonstration of polymorphism in the *CDKN2a* gene in human basal cell carcinomas. No sequence variation in exon 1β of the *p19*^{ARF} gene was found. Our results provide evidence of a significantly high occurrence of loss of heterozygosity for the 9p21–p22 locus; however, lack of *p16*$^{INK4a}/p19^{ARF}$ mutation suggests that these genes seem not to be implicated by mutational inactivation in the development of basal cell carcinoma. Other(s), yet unidentified, tumor suppressor gene(s) located in this locus may be related to this specific type of skin cancer.

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Basal cell carcinoma (BCC) of the skin is the most common neoplasia among the white population of the Western world (Miller and Weinstock, 1994; Zaphiropoulos *et al*, 1994; Gailani *et al*, 1996; D'Errico *et al*, 1997). More than 750,000 new cases are diagnosed annually in the U.S.A., and the incidence has been rising rapidly for the past several decades (Gloster and Brodland, 1996; Gailani and Bale, 1997). BCC is a keratinocyte-derived tumor that grows slowly, is locally invasive, rarely metastasizes (Quinn *et al*, 1994b), and is most often seen in elderly people (D'Errico *et al*, 1997). Solar ultraviolet radiation is a potent environmental DNA-damaging agent and a known inducer of skin cancer (Oro *et al*, 1997; Aszterbaum *et al*, 1999a, b). Chronic repeated exposure has been shown to be the primary cause of BCC and squamous cell (i.e., nonmelanoma) skin cancers (Armstrong and Kricker, 1996). Nevertheless, the potential part played by susceptibility genes in BCC risk is further supported by the observation that these tumors are also found on parts of the body not chronically sun exposed (D'Errico *et al*, 1997).

Allelic imbalance – loss of heterozygosity (LOH) and microsatellite instability (MI) – studies employ highly polymorphic microsatellite markers for the identification of sites in the genome with a high probability for the presence of candidate tumor suppressor genes (TSG) (Spandidos, 1986). A few studies exist on allelic imbalance in BCC showing that some human chromosomes exhibit LOH (Bare *et al*, 1992; Quinn *et al*, 1994b; Rees, 1994; Healy *et al*, 1995a; Holmberg *et al*, 1996; Johnson *et al*, 1996; Rees and Healy, 1996; Pont *et al*, 1997; Sikkink *et al*, 1997; Xie *et al*, 1997; Aszterbaum *et al*, 1998; Tabata *et al*, 1999).

The *p16* TSG (*INK4a/MTS-1/CDKN2a*) is a G_1-specific cell-cycle regulatory gene that is located on chromosome region 9p22.2 (Marx, 1994). The *p19*^{ARF} gene maps to the same chromosome region and encodes a protein that is derived in part from an alternative splicing of the *p16*, which has recently been shown to inhibit the *p16* cell cycle progression (Fargnoli *et al*, 1998). Another cyclin-dependent kinase inhibitor gene, the *p15*^{INK4b} is located on

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Abbreviations: MI, microsatellite instability; TSG, tumor suppressor gene; ARF, alternative reading frame; *CDKN2a*, cyclin-dependent kinase inhibitor 2a; INK4, inhibitor of CDK4.

chromosome region 9p21.2 and has a high structural and functional homology to the $p16^{INK4a}$ TSG (Kamb *et al*, 1994a). The $p16^{INK4a}$/$p19^{ARF}$ TSG are found altered in many primary tumors such as melanomas, carcinomas of the lung, bladder, breast, esophagus, head, and neck (Kamb *et al*, 1994a; Healy *et al*, 1995b, 1996a, 1998; Quélle *et al*, 1995; Reed *et al*, 1995; Wagner *et al*, 1998).

In order to determine the contribution of these genes to the development of another type of skin cancer, the BCC, we investigated the incidence of allelic imbalance (LOH and MI) in 67 BCC specimens from Greek patients using eight highly polymorphic microsatellite markers located close and around the $p16^{INK4a}$, $p19^{ARF}$, and $p15^{INK4b}$ TSG in chromosome arm 9p. LOH was found to be a frequent event exhibited in 40 of the 67 BCC specimens (60%), for the whole chromosome arm 9p and in 37 of the 67 BCC specimens (55%), for chromosome region 9p21–p22, with at least one microsatellite marker, whereas MI was rare, observed in only two of the 67 (3%) cases. The genetic alterations were also correlated with clinical and epidemiologic variables. Additionally, we screened for germline mutations 28 of our cases. The commonly shared by $p16^{INK4a}$ and $p19^{ARF}$ genes exon 2 and the alternatively spliced exon 1β of the $p19^{ARF}$ were screened. Two of the 28 cases were heterozygous for a previously described polymorphic sequence variant in exon 2 of the *CDKN2a* gene in melanoma kindreds (Ala148Thr) (Kamb *et al*, 1994b; Fargnoli *et al*, 1998).

MATERIALS AND METHODS

Tumor specimens and DNA extraction Sixty-seven sporadic BCC samples were obtained from the ''A. Sygros'' Hospital in Athens. The diagnosis of the samples was histologically confirmed. The specimens were stored at –70°C immediately after dissection until DNA extraction. A matched DNA control from blood was analyzed. DNA was extracted as previously described (Kiaris *et al*, 1994) and stored at –20°C until polymerase chain reaction (PCR) amplification.

The age of the patients ranged between 39 and 90 (average 68) y. Twenty-seven of the individuals were females and 40 were males. The head and neck for both sexes, the chest and shoulders for males, and the legs for females are characterized as sun exposed parts of the body (Gailani *et al*, 1996). Fifty of our 67 BCC specimens were located on the head and eight on the neck of the patients, two on the chest and three on the shoulders of five male patients, and four on the legs of four female patients. Thus, all studied BCC occurred on sun-exposed sites.

PCR amplification

Microsatellite analysis The DNA samples were examined for genetic alterations with 12 different highly polymorphic microsatellite markers (Research Genetics, Huntsville, AL). The selection of the chromosome region examined and of the markers used was based on previous studies on skin cancer that have shown they represent chromosomal locations exhibiting a variable degree of alterations. The microsatellite markers used and their exact location are shown in **Table I**. Chromosome region 9p21–p22 was screened with six markers which span a genetic distance of 7.529 cM.

PCR analysis were performed in a 50 μl reaction volume containing 500 ng of genomic DNA, 1 μM of each primer, 250 μM dNTP, 5 μl of 10 × buffer (670 mM Tris–HCl, pH 8.5, 166 mM ammonium sulfate, 67 mM magnesium chloride, 1.7 mg bovine serum albumin per ml, 100 mM β-mercaptoethanol, and 1% (wt/vol) Triton X-100) and 1 U of Taq DNA polymerase. The reactions were denatured for 3 min at 94°C and the DNA was subsequently amplified for 30 cycles at 94°C, 55°C and 72°C each step. The microsatellites of the PCR product were analyzed in a 7% polyacrylamide gel and silver stained.

Gels were scanned and the intensity of the bands corresponding to the microsatellite alleles was quantitated by an image analysis system. The analysis was performed at least twice and the results were highly reproducible. MI was diagnosed in case of an addition or deletion of one or more repeat units resulting in novel alleles. Allelic losses (LOH) were scored as significant decreases (more than 50%) in intensity of one allele relative to the other as determined from comparison of tumor and normal DNA from individuals who were heterozygous for the given locus. All the heterozygous cases were counted as well as those that were constitutionally homozygous (noninformative) for a marker in order to estimate the MI rate and the overall LOH rate, whereas in order to estimate the LOH rate for each marker separately only the heterozygous cases were counted.

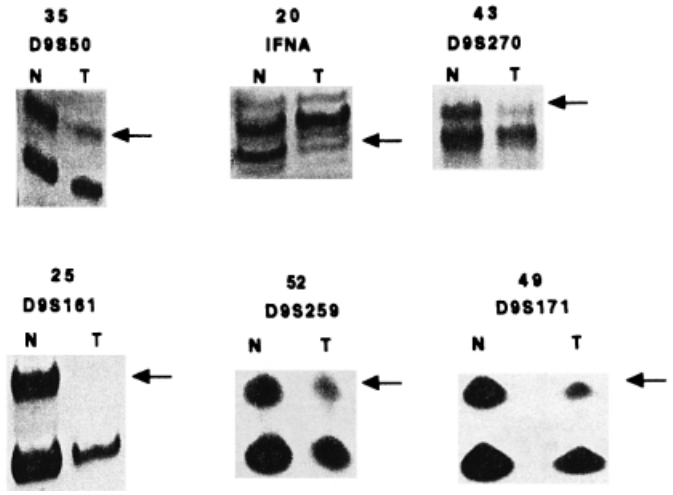

Figure 1. Representative examples of LOH detected in BCC. N, normal DNA; T, tumor DNA. *Arrows* indicate the position of a deleted allele. The faint bands in the position of the deleted alleles are interpreted as contamination by the adjacent normal tissue.

In a few cases a faint band appeared in the position of a deleted allele. This ''contamination'' is attributed to the presence of normal DNA derived by either peripheral blood or adjacent normal tissue. It has been suggested, however, that it is possible to obtain information regarding the heterogeneity of the tumor by quantitation of the allelic imbalance resulting from the PCR amplification of the microsatellite markers (Strachan and Read, 1996).

Statistical analysis of the results was performed with SPSS 6.0 (for Windows) and the chi-square analysis test. Statistical significance was set at $p < 0.05$.

Mutational analysis Exon 2 of both $p16^{INK4a}$ and $p19^{ARF}$ genes and the alternatively spliced exon 1β of the $p19^{ARF}$ were amplified by PCR from genomic DNA with the use of primers complementary to their flanking intron sequences. The sequences of the primers for exon 2 and exon 1β were identified from Fargnoli *et al* (1998) and synthesized. PCR was carried out under standard conditions as described above. Five per cent dimethyl sulfoxide was added to the reaction buffers for both nucleotide sequencing and PCR amplification. The reactions were denatured for 3 min at 94°C and the DNA was subsequently amplified for 40 cycles at 94°C, 60°C (for exon 1β), 61°C (for exon 2), and 72°C each step followed by a final extension step at 72°C for 20 min. The PCR products were resolved through 2% agarose gels, excised, and processed with the Wizard PCR Preps DNA purification Clean kit (Promega, Southampton, U.K.) to remove unincorporated primers and dNTP. The sequencing reaction contained: 4 μl Big Dye Terminator ready-reaction mix (PE ABI, Warrington, U.K.), 2 μl of cleaned PCR product and 1.6 pmol of sequencing primer in a total reaction volume of 10 μl. Reaction conditions were: 96°C for 10 s, 50°C for 10 s, 60°C for 4 min, 25 cycles. Sequencing products were precipitated with isopropanol to remove unincorporated dye terminators and resuspended in 4 μl of loading buffer (formamide:dextran sulfate/ethylenediamine tetraacetic acid, 5:1). Products were run on a 377 ABI PRISM automatic sequencer and analyzed with the Sequencing Analysis software (PE ABI). Both strands were sequenced for each PCR product from at least two independent PCR. The use of uncloned PCR products ensured representation of both *CDKN2a* alleles and minimized the possibility of errors that might be present within an individual PCR product. Base positions where the height of a secondary peak was about 50% that of the primary peak were marked as heterozygous and confirmed by analysis of both sense and anti-sense strands.

RESULTS

Sixty-seven BCC specimens were tested with a total of 12 polymorphic microsatellite markers located on chromosome regions 9p21–p22, 17p13, and 17q21. Overall, 69% (46 of 67) of the specimens examined displayed LOH in at least one of the microsatellite markers used, whereas only six of the 67 (9%) exhibited MI.

We used eight polymorphic microsatellite markers from chromosome arm 9p to determine the pattern and extent of

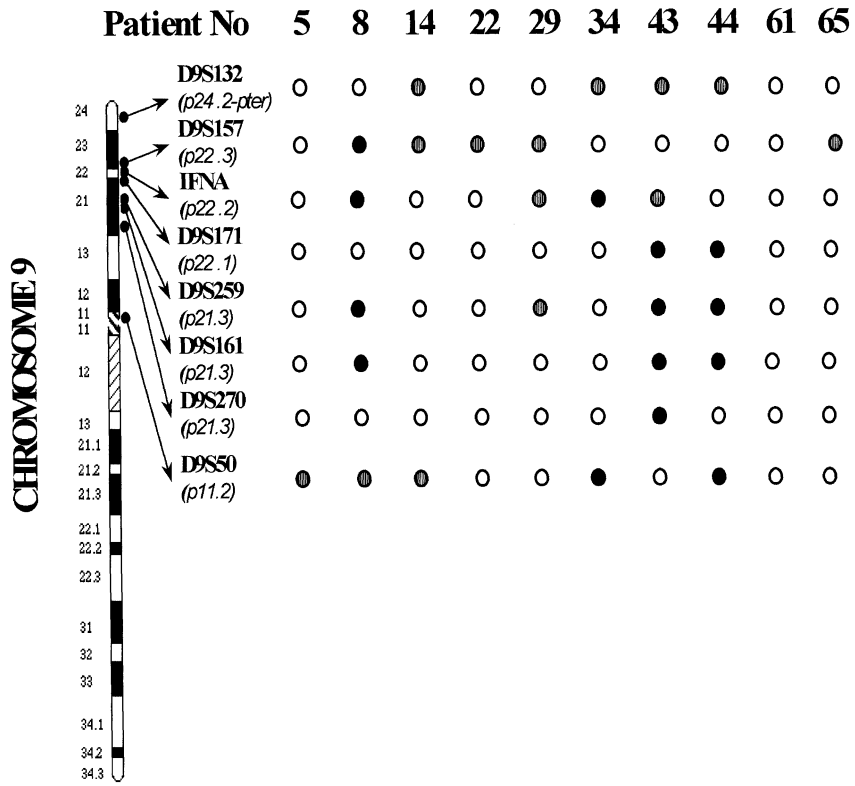

Figure 2. Location of the eight markers on chromosome arm 9p and pattern of LOH in 10 cases. (●, LOH; ○, heterozygosity; ●, non-informative or no data.)

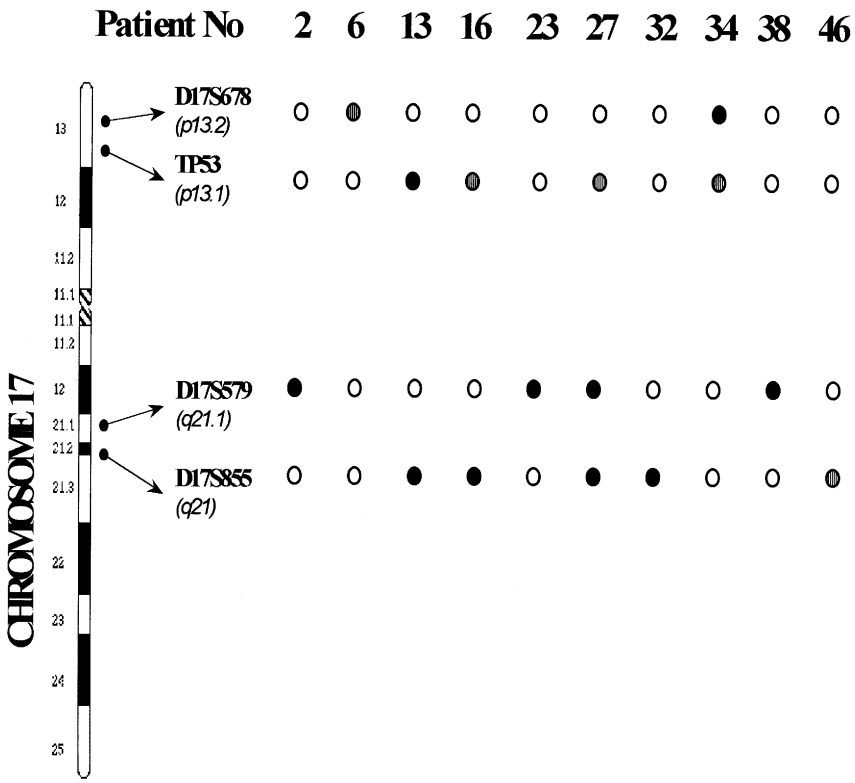

Figure 3. Location of the four markers on chromosome arms 17p and 17q and pattern of LOH in 10 cases. ●, LOH; ○, heterozygosity; ●, noninformative or no data.

chromosome loss in a series of 67 BCC. Representative examples of specimens with LOH are shown in **Fig 1**. Forty of the 67 BCC tumors (60%) exhibited LOH, with at least one microsatellite marker, which is the highest rate reported for chromosome arm 9p. Six of the eight microsatellite markers used reside on chromosome region 9p21–p22. Thirty-seven of the 67 BCC tumors (55%) exhibited LOH, with at least one microsatellite marker, which is the highest reported for region 9p21–p22. Ten of the 67 BCC tumors (15%) showed loss of more than one marker for region 9p21–p22, whereas 20 of the 67 BCC tumors (30%) showed loss of more than one marker for the whole 9p arm. MI was rare, observed in only two of the 67 (3%) cases. The most frequent incidence of

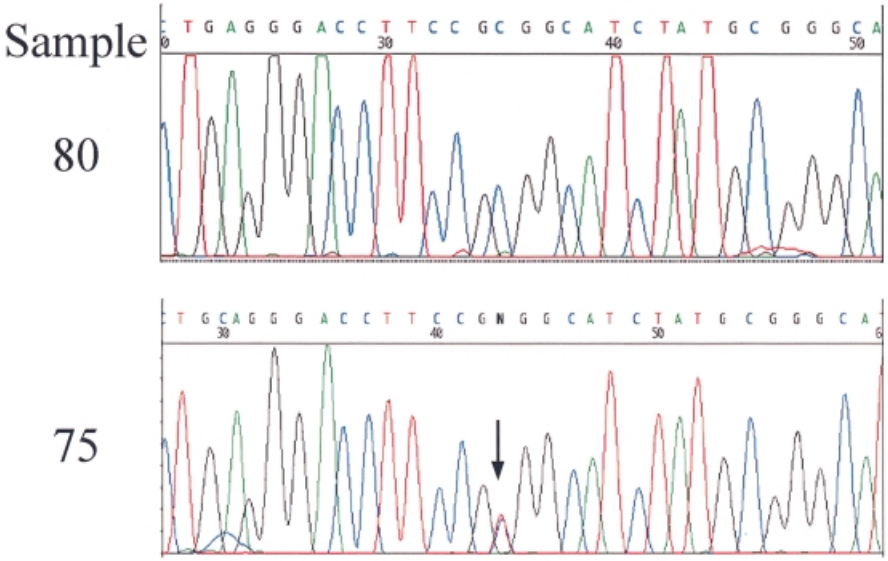

Figure 4. Comparison of sequencing (anti-sense strand) electrophorograms of two samples. Sample 75 is heterozygous for a G/A polymorphism (shown as a C/T in the anti-sense strand) whereas sample 80 lacks this polymorphism.

Table I. Location, frequency and percentage of LOH for the markers tested

Microsatellite marker	Chromosomal location	LOH (%)
D9S50	9p11.2	12/48 (25%)
D9S270	9p21.3	4/57 (7%)
D9S161	9p21.3	17/58 (29%)
D9S259	9p21.3	14/46 (30.5%)
D9S171	9p22.1	8/55 (15%)
IFNA	9p22.2	10/58 (17%)
D9S157	9p22.3	2/45 (4.5%)
D9S132	9p24.2 (pter)	9/40 (22.5%)
D17S855	17q21	15/52 (29%)
D17S579	17q21.1	10/60 (17%)
TP53	17p13.1	4/46 (9%)
D17S678	17p13.2	3/44 (7%)

LOH was found for marker D9S259 (14 of 46, 30.5%), closely followed by marker D9S161 (17 of 58, 29%), and marker D9S50 (12 of 48, 25%). Nine of 40 specimens exhibited LOH (22.5%) with marker D9S132 and 10 of 58 specimens exhibited LOH with marker IFNA (17%). For markers D9S171 (eight of 55, 14.5%), D9S270 (four of 57, 7%), and D9S157 (two of 45, 4.5%) the incidence of LOH was less frequent.

A total of four microsatellite markers situated on chromosome 17 were used in order to additionally test our BCC specimens. Markers D17S855 and D17S579 were situated on chromosome arm 17q, whereas markers TP53 and D17S678 on chromosome arm 17p. The overall percentage of LOH reached 34% (22 of 64) for 17q21 locus, whereas MI was rare, observed in only three of the 65 (4.5%) cases. Fifteen of 52 (29%) specimens displayed LOH for marker D17S855 and 10 of 60 (17%) for marker D17S579. For 17q13 locus, the overall LOH frequency reached only 11% (seven of 65) and MI was also rare observed in only three of the 67 (4.5%) cases. Four of 46 (9%) specimens displayed LOH for marker TP53 and three of 44 (7%) for marker D17S678.

The pattern of loss on chromosome arms 9p, 17p, and 17q for all the microsatellite markers tested in 10 randomly chosen cases is shown in **Figs 2** and **3**. The frequency of incidence of LOH for all the microsatellite markers used is shown in **Table I**. The pattern of loss on individual tumors is shown in **Table II**. No sample exhibited deletions of all informative markers tested; however, several tumors exhibited partial deletions of the 9p arm probably due to multiple mitotic recombinant genetic events.

No significance was found ($p = 0.96$, chi-square test) between the accumulation of LOH on chromosome arm 9p and the sex of the individuals. These data agree with a diversity of other studies that describe no significant correlation of LOH incidence with sex in a number of malignant and benign tumors (Mitsudomi *et al*, 1996; Detorakis *et al*, 1998). Similarly, no significance was found ($p = 0.22$, chi-square test) between the occurrence of LOH on chromosome arm 9p and the age of BCC onset. Furthermore, it was not possible to associate the existence of LOH with sun exposure by assessing the location of tumors on the body because all BCC studied occurred on sun-exposed sites.

***CDKN2a* polymorphisms – analysis of the $p19^{ARF}$ gene** We screened for germline mutations 28 of the cases. These were the cases that displayed LOH when tested with microsatellite markers IFNA, D9S171, and D9S259 which are situated closest to $p19^{ARF}$ and $p16^{INK4a}$ genes, and thus have a higher possibility of displaying a point mutation according to the ''Knudson's'' two-hit hypothesis (Knudson, 1971). The common to both genes exon 2 and the alternatively spliced exon 1β of the $p19^{ARF}$ were amplified by PCR from genomic DNA with the use of primers complementary to their flanking intron sequences (Fargnoli *et al*, 1998). We found a previously described polymorphic sequence variant in the *CDKN2a* gene, in both the tumor sample and blood DNA. A G→A transition at base 442 in exon 2 that results in an alanine to a threonine substitution at codon 148 (Ala148Thr) was detected in two of the 28 cases (**Fig 4**) (Kamb *et al*, 1994b; Fargnoli *et al*, 1998). Both cases were heterozygous for the given polymorphism. This polymorphism does not affect the $p19^{ARF}$ transcript despite the hypothesis that silent mutations in the $p16^{INK4a}$ gene caused by nucleotide substitutions could lead to mutations important in the $p19^{ARF}$ protein (Fargnoli *et al*, 1998). Additional, there was no sequence variation detected in any of the patients concerning the unique exon 1β of the $p19^{ARF}$ gene.

DISCUSSION

We found a significantly high incidence of LOH confined to chromosome arm 9p (40 of the 67 informative tumors; 60%) with at least one microsatellite marker. Our data confirm results of previous studies that reported a high incidence of LOH (four of

Table II. LOH in BCC specimens tested with 12 microsatellite markers

Patient no.	Microsatellite markers											
	D9S50	D9S270	D9S161	D9S259	D9S171	IFNA	D9S157	D9S132	D17S855	D17S579	TP53	D17S678
1	H[a]	H	H	LOH	H	H	H	H	H	H	H	N
2	H	H	H	LOH	H	H	H	H	H	LOH	H	H
3	MI	H	LOH	H	H	H	H	H	H	H	H	H
4	–[b]	H	H	–	H	–	–	–	–	H	–	N
5	N[c]	H	H	H	H	H	H	H	H	H	H	N
6	H	H	H	H	H	H	N	H	H	H	H	N
7	H	H	N	H	H	H	H	H	H	H	H	N
8	–	H	LOH	LOH	H	LOH	LOH	H	–	LOH	LOH	MI
9	LOH	H	LOH	N	LOH	H	H	H	H	H	LOH	N
10	H	H	N	H	–	H	N	LOH	H	H	H	H
11	N	H	H	H	H	H	N	H	H	H	H	H
12	–	–	LOH	LOH	H	H	–	LOH	LOH	LOH	–	N
13	–	–	H	LOH	–	H	H	LOH	LOH	H	LOH	H
14	N	H	H	H	H	H	H	N	H	H	N	H
15	H	H	H	–	H	H	H	H	H	H	H	H
16	H	H	H	H	H	H	H	N	LOH	H	N	H
17	N	H	H	H	H	H	H	–	H	H	N	H
18	H	H	H	LOH	H	H	H	–	H	H	H	H
19	LOH	H	LOH	H	H	LOH	H	N	–	H	–	H
20	H	H	LOH	–	N	LOH	H	LOH	–	H	–	H
21	LOH	LOH	N	H	H	H	H	H	H	H	H	H
22	H	H	H	H	H	H	N	H	H	H	H	H
23	LOH	LOH	N	H	H	H	–	H	H	LOH	H	H
24	H	H	N	H	LOH	H	H	H	H	H	H	H
25	H	H	LOH	H	H	H	–	LOH	H	H	N	H
26	H	H	H	N	N	H	H	N	H	–	N	H
27	LOH	H	N	LOH	H	H	H	N	LOH	LOH	–	H
28	H	H	N	–	–	H	N	–	–	H	–	H
29	H	H	H	N	H	–	N	H	H	H	H	H
30	H	H	H	H	N	H	H	N	H	H	H	N
31	–	H	–	N	H	H	H	H	H	H	H	H
32	H	H	LOH	H	–	–	H	H	LOH	H	H	H
33	H	H	H	N	–	H	H	H	H	H	H	H
34	LOH	H[a]	H	H	H	LOH	H	N	H	H	–	LOH
35	LOH	H	LOH	LOH	H	LOH	LOH	LOH	H	LOH	–	H
36	N[c]	H	LOH	N	–[b]	–	H	–	LOH	H	H	H
37	N	H	H	N	H	H	H	H	N	–	H	N
38	N	H	H	H	–	LOH	H	LOH	H	LOH	H	H
39	H	H	H	–	H	H	H	H	N	H	H	H
40	H	LOH	H	–	H	–	H	H	–	H	H	H
41	N	H	H	LOH	H	H	H	N	N	H	H	N
42	LOH	H	H	H	H	H	H	H	H	H	H	N
43	H	LOH	LOH	LOH	LOH	N	H	N	H	H	H	N
44	LOH	H	LOH	LOH	LOH	H	H	N	H	H	MI	LOH
45	H	H	H	H	LOH	H	H	LOH	H	H	H	H
46	N	H	H	LOH	H	H	H	H	N	H	H	H
47	H	H	LOH	H	H	H	H	–	LOH	H	H	N
48	H	H	H	H	H	H	MI	H	H	MI	MI	N
49	LOH	H	H	H	LOH	H	H	–	H	–	H	H
50	–	N	LOH	–	H	LOH	H	H	LOH	H	H	N
51	H	N	H	–	H	H	H	H	H	–	H	H
52	H	H	H	LOH	H	H	H	–	LOH	H	H	–
53	N	H	H	H	LOH	H	H	–	H	LOH	–	N
54	–	H	–	H	H	–	H	–	LOH	–	–	H
55	H	H	H	LOH	–	H	H	H	H	MI	–	H
56	H	N	LOH	N	LOH	LOH	N	H	LOH	MI	H	N
57	H	N	LOH	N	N	N	–	–	–	–	H	–
58	H	H	H	H	H	N	H	H	LOH	H	H	H
59	H	N	LOH	H	H	H	H	–	–	–	–	LOH
60	N	N	H	H	H	H	H	–	H	LOH	H	H
61	H	H	H	H	H	H	H	H	LOH	LOH	–	H
62	H	N	H	N	H	H	H	–	LOH	H	LOH	–
63	LOH	H	H	N	H	LOH	–	LOH	H	H	H	–
64	H	H	H	H	H	H	H	–	N	H	H	N
65	H	H	H	H	H	H	–	H	–	H	–	N
66	LOH	H	H	N	H	H	N	–	–	H	–	H
67	N	N	H	–	H	LOH	–	–	LOH	H	–	H

[a]H, heterozygosity; [b]–, no data; [c]N, noninformative.

eight informative BCC; 50%) (Gailani *et al*, 1992); however, they are contrary to other previous studies reporting a significantly lower incidence of LOH on the 9p arm. Two studies reported LOH in four of 33 informative tumors (12%) (Quinn *et al*, 1994b, c) and another reported no LOH of the short arm of chromosome 9 in any of the 19 informative BCC cases examined (Quinn *et al*, 1994a). We emphasize that, although the same chromosome arm was screened in all the above studies, differences exist in the markers used, in the number of specimens examined (67 in this study in contrast with eight in the study by Gailani *et al*, 1992; 33 in the studies by Quinn *et al*, 1994b and c; 19 in the study by Quinn *et al*, 1994a), as well as in the number of markers used (eight in our study instead of one in the studies by Quinn *et al*, 1994a and b and four in the study by Quinn *et al*, 1994c). The difference of our study with the above three studies, estimated by the chi-square test, is statistically significant: for both Quinn *et al* (1994b) and Quinn *et al* (1994c) the significance is $p < 0.00004$ and for Quinn *et al* (1994a) it is $p < 0.00002$.

Chromosome 17 was additionally tested in our BCC specimens with a total of four microsatellite markers. For the 17q21 locus, that harbors the *BRCA1* TSG, the overall frequency of LOH reached 34% (22 of 64), whereas for 17q13 locus, that harbors the *p53* TSG, the overall LOH frequency reached only 11% (seven of 65). Thus, the percentage of LOH for chromosome arms 17p (11%) and 17q (34%) is lower than that of chromosome arm 9p (60%) and of the 9p21–p22 region (55%). Furthermore, for markers D9S50 and D9S132, that are situated outside the 9p21–p22 area, the percentage of LOH is 25% (12 of 48) and 22.5% (nine of 40), respectively. These lower LOH frequencies compared with the LOH frequency confined to region 9p21–p22, suggests that the incidence of allelic imbalance is nonrandom and focused to this region.

The methodology applied in this study did not allow us to detect homozygous deletions; however, one cannot exclude the possibility that some of the areas of apparent retention of heterozygosity could be due to homozygous deletions and thus we cannot rule it out as a possibility.

Apart from the allelic imbalance analysis, we also performed mutational analysis and screened 28 of the cases for germline mutations. These were the cases that displayed LOH when tested with microsatellite markers IFNA, D9S171, and D9S259, which are situated the closest to $p16^{INK4a}$ and $p19^{ARF}$ TSG. Most of the mutations found in melanoma kindreds (Flores *et al*, 1997; Fargnoli *et al*, 1998; MacKie *et al*, 1998) and squamous cell carcinomas (Kubo *et al*, 1997) are detected in exon 2. In order to cover the entire coding region of the $p19^{ARF}$ gene we also screened exon 1β, although no evidence of mutation altering only $p19^{ARF}$ exist in melanomas (Flores *et al*, 1997; Fargnoli *et al*, 1998) or in squamous cell carcinomas (Kubo *et al*, 1997). We did not screen the $p15^{INK4b}$ gene because, to date, no mutations of this gene have been reported in melanoma kindreds (Flores *et al*, 1997; Platz *et al*, 1997). Contrary to Kubo *et al* (1998) who described the lack of mutation in the INK4a locus in BCC, we have found a polymorphic sequence variant in the *CDKN2a* gene that other groups previously described in melanoma kindreds. In both the tumor sample and blood DNA a G→A transition at base 442 in exon 2 which results in an alanine to a threonine substitution at codon 148 (Ala148Thr) was detected in two of the 28 cases examined. Both of these cases were heterozygous for the given polymorphism (Kamb *et al*, 1994b; Fargnoli *et al*, 1998). The result of this polymorphism is a different amino acid, and probably a protein with a different conformation, something that could have broader consequences on its biologic function. Nevertheless, the $p19^{ARF}$ transcript is not affected by this polymorphism and, in addition, no sequence variation was detected in any of the patients concerning the unique exon 1β of the $p19^{ARF}$ gene, supporting the concept that $p19^{ARF}$ TSG does not seem to play a significant part in skin tumors, in general (Flores *et al*, 1997; Fargnoli *et al*, 1998; MacKie *et al*, 1998).

The sequencing analysis that was performed showed that in these two cases both copies of the $CDKN2a/p16^{INK4a}$ gene were intact. The $CDKN2a/p16^{INK4a}$ gene is a relatively small gene that contains only three exons. None of the microsatellite markers that exist in this chromosome region are located within this gene. Thus, although allelic loss is detected on chromosome region 9p21–p22, it is not confined to, but close and around this gene, supporting the fact that the $CDKN2a/p16^{INK4a}$ gene remains intact. It is not common for small size genes to demonstrate mutations. In a study reported by Healy *et al* (1996b) concerning sporadic melanoma only one of the 26 cases of sporadic cutaneous melanoma contained a mutation. The fact that this tumor also exhibited allelic loss of 9p adds support to the theory that $p16^{INK4a}$ is important in the development of sporadic cutaneous melanoma. The low frequency of mutations of $p16^{INK4a}$ gene coupled with LOH of 9p, however, suggests that another TSG near this locus may exist and be involved in some cases of sporadic melanoma (Healy *et al*, 1996b); a suggestion that is consistent with our results for BCC tumors. From the results of our study we can conclude that the mutational inactivation of $p16^{INK4a}$ gene does not seem to be the major event in BCC development. Furthermore, the $p16^{INK4a}$ gene is not found frequently inactivated by mutation in a number of tumors (Healy *et al*, 1996b; Liggett and Sidransky, 1998). Nevertheless, one cannot yet exclude the possibility of the involvement of $p16^{INK4a}/p19^{ARF}$ genes in BCC tumorigenesis, through other alterations such as aberrant expression, as has been reported for other TSG in other types of cancer (Sourvinos and Spandidos, 1998).

An attempt was made to correlate the existence of LOH on chromosome region 9p21–p22 with sunlight exposure, as assessed by the location of BCC tumors on the body. Gailani *et al* (1996) found no association between sunlight exposure and the existence of LOH on chromosome region 9q22.3–q31, which harbors the naevoid basal cell carcinoma syndrome TSG. As all our BCC specimens came from sun-exposed parts of the body it was not possible to associate the existence of LOH with sun exposure by assessing the location of tumors on the body. Besides the attempt of the association of sun exposure with LOH, mutational alterations are also correlated with ultraviolet irradiation. In a recent study by Soufir *et al* (1999) the presence of $p16^{INK4a}$ ultraviolet-induced mutations in nonmelanoma skin cancer (including BCC), which is demonstrated for the first time, supports the hypothesis that $p16^{INK4a}$, as well as *p53* TSG are involved in skin carcinogenesis probably in two independent pathways. In addition according to Pollock *et al* (1995) indicative of a possible role of ultraviolet irradiation in the mutagenesis of $p16^{INK4a}$ gene in melanoma cell lines are C:G to T:A transitions detected in this gene. The same base transition detected in both our BCC cases and the fact that all our specimens came from the Greek population, which is characterized by a very high degree of lifetime and recreational sun exposure, allow us to hypothesize that sunlight exposure may be implicated in BCC development. Our results showing MI in six of the 67 tumors examined (9%) agree with the results of Quinn *et al* (1995) that changes in microsatellite length are uncommon in nonmelanoma and melanoma skin cancers, occurring in approximately 5% of the tumors examined.

In conclusion, the high incidence of LOH confined to chromosome region 9p21–p22 indicates that important TSG for the development of BCC may be located on this region. The absence of mutation of $p16^{INK4a}/p19^{ARF}$ genes in cases of sporadic BCC coupled with LOH in the 9p21–p22 region implies that these genes do not seem to be mutationally inactivated; however, other(s) yet unidentified TSG(s) located near this locus may be involved in sporadic BCC development.

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