Low Frequency of Loss of Heterozygosity At the Nevoid Basal Cell Carcinoma Locus and Other Selected Loci in Appendageal Tumors

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Previous studies of loss of heterozygosity (LOH) have revealed distinct patterns of allelic loss in some skin tumors. In basal cell carcinomas (BCCs) loss of heterozygosity is virtually restricted to chromosome 9, whereas in squamous cell carcinomas (SCCs) and actinic keratoses loss is more widespread involving chromosomes 3, 9, 13, and 17. Because there are histological similarities between BCCs and some appendageal tumors, and because some lines of evidence suggest that BCCs are appendageal in origin, we carried out a limited allelotype in 41 appendageal tumors. The overall frequency of allelic loss was low (4 out of 247 informative loci; 1.6%). LOH was seen in a proliferating trichilemmal cyst (17p), a sebaceous epithelioma (17q), an eccrine porocarcinoma (17q), a trichoepithelioma (9q), and in two basal cell carcinomas showing eccrine or granular cell differentiation that were originally misdiagnosed (9q). The pattern of loss in this mixed group of appendageal tumors shows differences from both BCCs and SCCs, and further emphasizes the unique genetic profile and behavior of BCCs. The finding of 9q loss in BCCs with eccrine or granular cell differentiation shows that 9q loss occurs in different histological subtypes of BCCs.

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Current models of human carcinogenesis emphasize the causal relation between the accumulation of genetic abnormalities and the clinical behavior of a neoplasm (Yokota and Sugimura, 1993). Probably the best example is colorectal cancer where work by Vogelstein and others (Fearon and Vogelstein, 1990) has shown that the stages of tumor progression as defined histopathologically correlate with the accumulation of abnormalities in oncogenes and tumor suppressor genes. Although oncogenes were the first genes identified in human cancer, and therefore earlier work focused on their characterization, recent attention has centered on the role of tumor suppressor genes in human neoplasia (Ponder 1988; Sager, 1989; Fearon and Vogelstein, 1990; Yokota and Sugimura, 1993). Evidence implicating tumor suppressor genes in cancer pathogenesis comes from a number of sources. First, cell fusion studies between normal and malignant cells suggested that certain chromosomes possessed tumor suppressing activity, and that this characteristic was recessive (Anderson and Stanbridge, 1993). Second, based on observations of the epidemiology of retinoblastoma, Knudson (1971) proposed that some inherited human malignancies may be due to an inherited mutation in one copy of a tumor suppressor followed by a second hit to the other allele. This hypothesis suggested that similar (recessive) genes may be involved in inherited and sporadic cancers. Subsequent work has vindicated this hypothesis for a number of different tumor types (Ponder, 1988). It is of interest to cutaneous biologists that the germ of this idea as applied to mouse skin cancers was originally suggested in the 1940s (Charles and Luce-Clausen, 1942).

In epithelial neoplasms, defects in putative tumor suppressor genes seem especially common and outnumber the oncogenes implicated in these cancers (Fearon and Vogelstein, 1990; Yokota and Sugimura, 1993). This may in part relate to the technical strategies adopted to identify new cancer-causing genes (Ponder, 1988; Sager, 1989). Although tumor suppressor genes may be inactivated in a number of different ways, including binding to viral proteins or products of other genes (Lane, 1994), a particularly common mechanism is mutation of one allele followed by loss of the remaining allele (Ponder, 1988; Sager, 1989). This deletion of genetic material results in loss of heterozygosity (LOH) when alleles are examined using polymorphic markers, making it possible to screen chromosomal areas of interest using polymerase chain reaction LOH-based assays.

Examination of the pattern of allele loss across all the autosomes in tumors from different organs shows that although there are many similarities, perhaps reflecting the important cellular function of certain key genes such as p53 and retinoblastoma in skin, there are clear differences between basal cell and squamous cell carcinomas (Quinn *et al*, 1994a; Rees, 1994). Squamous cell carcinomas (SCCs) show a high frequency of allele loss at a number of loci including 3p, 9p, 9q, 13, and 17p (Quinn *et al*, 1994a). By contrast, and uniquely among human epithelial cancers that frequently undergo p53 mutation (van der Riet *et al*, 1994), the majority of

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Abbreviations: LOH, loss of heterozygosity; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; NBCCS, nevoid basal cell carcinoma syndrome locus.

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Table I. Low Frequency of Loss of Heterozygosity At Selected Loci in Cutaneous Appendageal Tumors[a]

Tumor	(Number)	Chromosome Arm[b] 1q	3p	9p	9q	13q	17p	17q	19q
Eccrine poroma	(7)	0/7	0/7	0/4	0/6	0/7	0/6	0/3	0/4
Pilomatricoma	(5)	0/5	0/5	0/4	0/4	0/4	0/4	0/5	0/4
Trichilemmoma y	(2)	0/1	0/2	0/2	0/1	0/2	0/1	0/1	0/1
Trichoepithelioma	(1)	ND[c]	ND	0/1	1/1	ND	ND	ND	0/0
Clear cell hydroadenoma	(4)	0/3	0/3	0/3	0/2	0/3	0/3	0/4	0/3
Eccrine spiradenoma	(3)	0/2	0/1	0/1	0/2	0/2	0/2	0/1	0/1
Cylindroma[d]	(12)	0/7	0/9	0/11	0/12	0/9	0/9	0/9	0/8
Acrosyringeal nevus	(1)	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Apocrine cystadenoma	(1)	0/1	0/1	0/0	0/1	0/1	0/1	ND	0/1
Apocrine ductadenoma	(1)	0/1	0/1	0/1	0/1	0/1	0/0	0/1	0/1
Proliferating trichilemmal cyst	(1)	0/1	0/1	0/1	0/0	0/1	1/1	0/1	0/1
Sebaceous epithelioma	(1)	0/1	0/1	0/1	0/0	0/1	0/1	1/1	0/0
BCC[e]	(2)	0/2	0/2	0/2	2/2	0/2	0/2	0/2	0/2
Eccrine porocarcinoma	(1)	0/1	0/1	0/1	0/0	0/1	0/1	1/1	0/0
Merkel cell carcinoma	(1)	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/0

[a] Number of tumors showing loss of heterozygosity/number of tumors informative.
[b] Microsatellite markers used are given in the text.
[c] *Not done.*
[d] Obtained from three patients, two of which from the same kindred of dermal cylindromatosis.
[e] Granular cell BCC (20) and BCC showing eccrine differentiation (21).

deletions that occur in basal cell carcinoma center around one particular locus, the nevoid basal cell carcinoma syndrome locus (NBCCS) at 9q22–31 (Gailani *et al*, 1992; Quinn *et al*, 1994b; van der Riet *et al*, 1994). Although it is tempting to expect differences between BCCs and SCCs because SCCs may behave more aggressively, preinvasive lesions such as actinic keratoses show a rate of allelic loss similar to, if not higher than that of SCCs (Rehman *et al*, 1994). It is for these reasons that we studied the pattern of allelic loss in appendageal tumors. Many of these tumors show histological similarities to, and overlap clinically with BCCs (Lever, 1948a; Lever and Schaumburg-Lever, 1990). Conversely, there is evidence that BCCs may be derived from the adnexal structures (Lever, 1948b; Pinkus, 1953). We were therefore interested to see whether the pattern of allele loss in appendage tumors would resemble that seen in BCCs or other cutaneous neoplasms such as SCCs.

MATERIALS AND METHODS

Forty-three tumors from 34 patients with a primary pathological diagnosis of any appendageal tumor were retrieved from the pathology files of Kanazawa University Hospital, Japan, and the Royal Victoria Infirmary, Newcastle, UK. Subsequently, hematoxlin and eosin-stained slides of all the tumors were reviewed independently by two expert dermatopathologists (M.T. and K.H.) without knowledge of the LOH data, and a revised pathological diagnosis was made for each tumor. Two patients from the same kindred presented with multiple cylindromas and eccrine spiradenomas.

After a representative paraffin block was selected for each tumor, 15-μm sections were cut, dewaxed, and microdissected on an inverted microscope. Tumor DNA and corresponding normal DNA was isolated by proteinase K digestion and phenol-chloroform extraction (Jackson *et al*, 1992). In some cases, tumor DNA were directly isolated from snap-frozen samples and control DNA was obtained from venous blood. Analysis of LOH was carried out by polymerase chain reaction amplification of microsatellite polymorphisms as described previously (Quinn *et al*, 1994a,b). Briefly, polymerase chain reaction was performed with approximately 100 ng of template DNA with 200 mM deoxynucleotide triphosphates, 1 pmol of each oligonucleotide primer, one of which was end-labeled with [γ-^{32}P]ATP, and 1 unit of *Taq* DNA polymerase (Biotaq; Bioline, UK). Amplification consisted of 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min 72°C with a final 10-min extension at 72°C. The microsatellite oligonucleotide primers used were D1S212 (1q), D3S1293 (3p), D9S162 (9p), D9S171 (9p), D9S176 (9q), D9S197 (9q), D9S160 (9q), D13S170 (13q), D17S796 (17p), D17S785 (17q), and D19S255 (19q), all obtained from Research Genetics (Huntsville, AL). Polymerase chain reaction products were separated by electrophoresis through 6% acrylamide gels. Because sufficient DNA was available from the two tumors showing 9q loss, detailed deletion mapping on chromosome 9 as well as a allelotype of all 39 autosome arms were carried out for these two tumors using the following markers: D9S162, D9S171, D9S169, D9S161, D9S163 (9p), D9S165, D9S166, D9S152, D9S197, D9S180, D9S173, D9S176, D9S172, D9S160, D9S174,D9S158 (9q), D1S201 (1p), D1S304, D1S212 (1q), D2S166, D2S149 (2p), D2S163, (2q), D3S1268 (3q), D4S394 (4p), D4S402 (4q), D5S419 (5p), D5S410 (5q), D6S299 (6p), D6S262 (6q), D7S481 (7p), D7S495 (7q), D8S261 (8p), D8S257 (8q), D10S226 (10p), D10S185 (10q), D11S922 (11p), D11S910 (11q), D12S98 (12p), D12S86 (12q), D14S73 (14q), D15S118 (15q), D16S414 (16p), D16S422 (16q), D18S59 (18p), D18S70 (18q), D19S216 (19p), D20S104 (20p), D20S100 (20q) D21S262 (21q), and D22S283 (22q).

RESULTS

Histopathological re-review on hematoxlin and eosin sections identified 39 benign appendageal tumors (seven eccrine poromas, five pilomatricomas, two trichilemmomas, one trichoepithelioma, four clear cell hidradenomas, three eccrine spiradenomas, 12 cylindromas, one acrosyringeal nevus/eccrine syringofibroadenoma, one appocrine cystadenoma, one appocrine ductadenoma, one proliferating trichilemmal cyst, one sebaceous epithelioma), two BCC variants (one granular cell BCC (Barr and Graham, 1979) and one BCC with eccrine differentiation (Freeman and Winkelmann, 1969)), and two appendageal carcinomas (one eccrine porocarcinoma and one Merkel cell carcinoma) **(Table I)**. The two histological variants of BCCs were included in this study because their original pathological diagnosis had been trichilemmoma and clear cell hidradenoma, respectively, and because examination of the pattern of allele loss in these types of BCC has not been reported before.

The overall frequency of LOH on the eight chromosome arms examined was low with only 4 of 41 tumors showing loss (excluding the two BCCs). No appendage tumors showed loss at more than one locus **(Table I)**. The rare variants of BCC (granular cell BCC and BCC with eccrine differentiation), and one trichoepithelioma showed LOH of chromosome arm 9q with the retention of 9p. Full allelotype analyses and the detailed deletion mapping of chromosome 9 were carried out for these two BCC variants. In the granular cell BCC, 30 out of 39 chromosome arms were informative and allelic loss was only seen for loci on 9q and 4q. Deletion mapping of chromosome 9 revealed a deleted region telomeric to D9S166 including D9S197, D9S180, D9S176, and D9S160. In the BCC with eccrine differentiation, no allelic loss except for chromosome 9q was identified for 35 informative chromosome arms. Deletion mapping of chromosome 9 for this tumor showed retention of D9S152 which lies between 9q13 and 9q22.1 and loss of

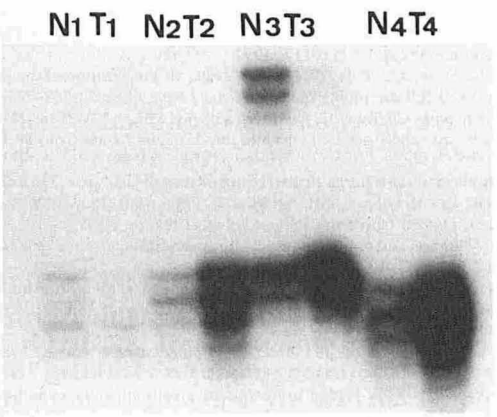

Figure 1. Loss of heterozygosity analysis in appendageal tumors using microsatellite polymorphism D17S785 (17q). Polymerase chain reaction products from normal (*N*) and tumor (*T*) DNA. Allelic losses are seen in an eccrine porocarcinoma (*T1*) and a sebaceous epithelioma (*T3*). Two pilomatricomas (*T2* and *T4*) show no loss.

D9S197, which is a proximal flanking marker for the BCCNS locus (Goldstein *et al*, 1994), as well as loss of D9S173. Unfortunately, these analyses could not be carried out for the trichoepithelioma because of lack of sufficient tumor DNA.

Isolated loss of chromosome arm 17q with retention of 17p was observed in a sebaceous epithelioma and an eccrine porocarcinoma **(Fig 1)**. A proliferating trichilemmal cyst showed isolated 17p loss. No allelic loss was identified in the one case of Merkel cell carcinoma. In view of the reported association between pilomatricoma and myotonic dystrophy (Chiaramonti and Gilgor, 1978) (which maps to chromosome 19q (Smeets *et al*, 1991)), the frequency of LOH at D19S255 was investigated in pilomatricomas and other cutaneous appendageal tumors. None of the 27 informative tumors, including four pilomatricomas, showed allelic loss.

DISCUSSION

We have examined 41 appendageal tumors for LOH at loci on chromosome arms implicated in either BCCs or SCCs (Gailani *et al*, 1992; Quinn *et al*, 1994a), and on chromosome 19q (Smeets *et al*, 1991) because of the reported association between pilomatricomas and myotonic dystrophy (Chiaramonti and Gilgor, 1978). We found LOH to be uncommon: one trichoepithelioma showed 9q loss (but see below); one proliferating trichilemmal cyst showed loss of 17p; both a sebaceous epithelioma and an eccrine porocarcinoma showed loss of 17q. In interpreting the results there are a number of factors that need to be considered. First, although samples were microdissected from visible contaminating tissue, it is impossible to exclude in all cases that LOH may have been missed because of the presence of nontumor cells. Similar studies on equally small lesions where LOH was detected (Quinn *et al*, 1994a; Rehman *et al*, 1994), however, suggest that this is unlikely to have been a major problem. Second, the finding of LOH, without further functional experiments in the appropriate cells, does not prove the presence of an underlying tumor suppressor gene (Sager, 1989). Alternatively, inactivation of a tumor suppressor gene may have occurred by other mechanisms such as point mutation (Fearon and Vogelstein, 1990). Small deletions are likely to have been missed by the present study.

BCCs are unusual both in terms of their clinical behavior and their genetics. Clinically, while they may be quite indolent and rarely metastasize, they are invasive and locally destructive (Miller, 1991). Their genetics is unusual because, unlike many other epithelial tumors which show frequent p53 mutations, they rarely show allelic loss, with the exception of the chromosome 9, which harbors the NBCCS locus (Gailani *et al*, 1992; Quinn *et al*, 1994a,b; Rees, 1994; Rehman *et al*, 1994; van der Riet *et al*, 1994). Loss encompassing this locus occurs in up to 70% of sporadic tumors (Quinn *et al*, 1994a). Various lines of evidence suggest that BCCs may be appendageal in origin (Lever, 1948b; Pinkus, 1953), and because of both clinical and histological overlap between BCCs and some adnexal tumors (Lever, 1948a; Lever and Schaumburg-Lever, 1990), we were interested to examine whether the loss of the NBCCS locus occurred in adnexal tumors. Any similarities between appendageal tumors and BCC might reflect a number of different mechanisms. For instance, if different tumor types are derived from a common normal progenitor cells, then the pathways of aberrant differentiation leading to neoplasia may show similarities. Second, appendageal tumors and BCCs might share an early common neoplastic stage before their pathways diverge, and third, irrespective of their cellular origins, loss of the NBCCS locus might restrict the subsequent phenotype of an epidermal keratinocyte tumor.

Using markers that are close to the NBCCS locus (Goldstein *et al*, 1994; Wicking *et al*, 1994) and are known to be deleted at high frequency in sporadic BCCs (Quinn *et al*, 1994b; van der Riet *et al*, 1994), allele loss was initially only seen in three of the tumors we screened. Deletion mapping in these cases showed that the interstitial deletions were centered around the NBCCS locus (Goldstein *et al*, 1994; Wicking *et al*, 1994). Subsequent to the molecular analysis, however, expert pathological review of two of these cases suggested that they were in fact BCCs with either granular cell (Barr and Graham, 1979) or eccrine cell differentiation (Freeman and Winkelmann, 1969) respectively, rather than a trichilemmoma and a clear cell hidradenoma. The remaining case, that of a trichoepithelioma that showed loss at the NBCCS locus, should be interpreted carefully. This was a solitary trichoepithelioma excised from a patient who had received treatment for multiple BCCs. As the differentiation of trichoepithelioma from keratotic BCC on histological grounds alone is very difficult or even impossible (Lever and Lever-Schaumburg, 1990), we cannot exclude the possibility that the tumor was not a trichoepithelioma. It is worth noting that an association between trichoepitheliomas and BCCs has been reported in patients with Bazex syndrome (Bazex *et al*, 1966) (which maps to Xq24-q27 (Vabres *et al*, 1995)) and the Rombo syndrome (Michaelsson *et al*, 1981). The almost complete absence of loss of 9q in appendageal tumors suggests a clear genetic difference from BCCs. It is not, however, very helpful in further examining the relation between appendageal and basal cell carcinoma origins. Given that the two tumor types are often similar in size, if the tumors do share a common early stage, then loss of 9q must occur after their pathways of differentiation have separated. It is interesting to note that the two histological variants of BCCs accidentally included in this study showed LOH of chromosome 9q. Full allelotype and fine deletion mapping of chromosome 9 in these tumors revealed that LOH was restricted to chromosome 9q with the deleted area encompassing the NBCCS locus (Goldstein *et al*, 1994; Wicking *et al*, 1994). This result clearly indicates that these two cases of BCC variant have the same characteristic genetic change of ordinary BCCs (Gailani *et al*, 1992; Quinn *et al*, 1994a,b; Rees, 1994; van der Riet *et al*, 1994). Previous studies have not examined the relationship between histological type and the pattern of genetic changes in BCCs (Gailani *et al*, 1992; Quinn *et al*, 1994a,b; Rees, 1994; van der Riet *et al*, 1994), and our result suggests that chromosome 9q loss occurs in a variety of different histological types of BCC.

Dermal cylindromatosis is an autosomal dominant disorder, and recent genetic linkage analysis reported the localization of the gene to chromosome 9p 12–13 between D9S161 and interferon gene cluster (Wooster R, Mangion J, Quirk Y, Ford D, Easton DF, Chapman P, Burn J, Weaver-Feldhaus J, Kamb A, Ponder BAJ, Stratton MR: Localization of the gene for cylindromatosis (turban tumor syndrome) to chromosome 9p12–13. *Am J Hum Genet* 55(Suppl):A207, 1994, abstr.). All of the tumors excised from our two patients belonging to the same kindred of this disorder, however, did not show LOH for 9p markers D9S162 and D9S171, although these two microsatellite markers locate slightly telomeric

to the reported gene locus. Trichoepitheliomas and eccrine spiradenomas are commonly associated with dermal cylindromatosis (Welch *et al*, 1968; Rasmussen, 1969), but no loss of chromosome 9p was observed in the trichoepithelioma and four eccrine spiradenomas examined. The absence of LOH at this locus does not exclude an underlying predisposition gene, however, as not all inherited cancer-causing genes are accompanied by LOH in the primary tumors (Ponder, 1988; Fearon and Vogelstein, 1990).

The finding of LOH for 17q in a sebaceous epithelioma and an eccrine porocarcinoma, and loss of 17p in a proliferating trichilemmal cyst demonstrates that the lesions are clonally derived (otherwise one would not see clear evidence of LOH). Chromosome 17 harbors a number of candidate tumor suppressor genes including BRCA1 (17q) (Hall *et al*, 1992) and p53 (17p) (Nigro *et al*, 1989), and loss of 17p and 17q is frequently seen in SCCs (Quinn *et al*, 1994a). With respect to the proliferating trichilemmal cyst, sufficient DNA was available to allow sequencing of the p53 gene, which showed a missence mutation in exon 8: this suggests that in this particular instance p53 was the target tumor suppressor gene on chromosome 17, although others may be involved (Wales *et al*, 1995).

A final point relates to the low frequency of loss seen at any locus in the tumors studied. Because not all autosomes were examined, it is possible that allelic loss may in fact be higher. The loci studied were chosen because of their frequent involvement in other forms of skin cancer and epithelial malignancies. Mapping of any of the inherited syndromes involving appendageal tumors to other autosomes would invite further scrutiny of these regions in sporadic tumors. This notwithstanding, the low rate of allelic loss is perhaps in keeping with the behavior of most of the neoplasms examined. Many appendageal tumors are clinically benign, although it is worth noting that this is not true for some of the tumors studied, including the sebaceous epithelioma, Merkel cell carcinoma, and eccrine porocarcinoma (Lever and Schaumburg-Lever, 1990), all of which showed no or only minimal loss. In other cancer systems, early lesions which are clinically benign may show LOH (Lalle *et al*, 1994), and in skin the relation between loss and behavior is not straightforward, with preinvasive lesions such as actinic keratoses showing as high or higher rate of loss than SCCs (Rehman *et al*, 1994). Our results suggest that other explanations and mechanisms of aberrant growth control may need to be examined to explain the behavior of these neoplasms.

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