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Analysis of *APC* Allelic Imbalance/Loss of Heterozygosity and APC Protein Expression in Cutaneous Squamous Cell Carcinomas

SARAH E. GRAY[1], ELAINE W. KAY[1], MARY LEADER[1] and MOHAMED MABRUK[2]

[1]*Pathology Department, Royal College of Surgeons in Ireland and Beaumont Hospital, Dublin, Ireland;*
[2]*PAPRSB, Institute of Health Sciences, University of Brunei Darussalam, Bandar Seri Bagawan, Brunei Darussalam*

Abstract. *Background: The adenomatous polyposis coli (APC) gene is a tumor suppressor gene which is mutated in the hereditary disease, familial adenomatous polyposis (FAP). Somatic mutations of the APC gene have also been identified in the majority of sporadic colorectal carcinomas, and mutation of the APC gene appears to be an early step in the initiation of colon cancer. Loss of heterozygosity (LOH) of APC has been described in a variety of other cancer types, including renal cell carcinoma, gastric cancer, non-small cell lung cancer, endometrial cancer and oral squamous cell carcinomas (SCC). Aim: To determine the role played by APC gene in the genesis of cutaneous SCC. Materials and Methods: Allelic imbalance/loss of heterozygosity (AI/LOH) was examined in twenty-two histologically confirmed cutaneous squamous cell carcinomas (SCC) using microsatellite markers, proximal to the APC gene. Immunohistochemical analysis of APC protein expression was also examined in the cutaneous SCC. Results: AI/LOH was detected in 60% of the SCC samples using D5S346 marker (proximal to the APC gene). Ninty-five percent of the SCC samples showed positive reduced APC expression, however the localization of the APC protein was abnormal. Conclusion: The abnormal expression of APC suggests that APC gene may play a role in cutaneous SCC development.*

Squamous cell carcinoma (SCC) of the skin is one of the most common malignancies among Caucasians worldwide, particularly the elderly (1). It is estimated that there are between 400,000-600,000 cases of cutaneous SCC worldwide each year (2). Exposure to UV radiation is the major environmental risk factor resulting in DNA damage and contributing to the development of cutaneous SCC in the Caucasian population, with fair-skinned individuals at greatest risk (3).

The human *APC* gene is a tumor suppressor gene located on the long (q) arm of chromosome 5. More than 700 mutations in the *APC* gene have been identified in families with the classic and attenuated types of familial adenomatous polyposis (FAP). Most of these mutations lead to the production of an abnormally short, nonfunctional version of the APC protein. This short protein cannot suppress the cellular overgrowth that leads to the formation of polyps, which can become cancerous (4). Somatic mutations of the *APC* gene have also been identified in the majority of sporadic colorectal carcinomas, and mutation of the *APC* gene appears to be an early step in the initiation of colon cancer (5, 6).

Loss of heterozygosity is a widely used molecular approach for both hereditary and sporadic cancer to identify common regions of chromosome loss in tumors of the same type and thus identify possible tumor suppressor genes involved in tumor formation and can also highlight the presence of novel tumor suppressor genes (7). LOH analysis involves the use of genetic markers within or flanking a chromosomal region of interest. Comparison of a genetic marker isolated from the normal DNA of an individual who is a heterozygote for that marker with the corresponding genetic marker isolated from the tumor DNA of the same individual is used to detect LOH. LOH of the genetic marker in the tumor DNA indicates loss of an allele of this marker, and most likely loss of the chromosomal region of interest, if not the entire chromosome (8).

LOH of *APC* has been described in a variety of other cancer types, including renal cell carcinoma, gastric cancer, non-small cell lung cancer, endometrial cancer and oral SCC (9-14). In this study, in addition to analysis of LOH in the region of *APC* gene, expression of the *APC* protein in cutaneous SCC was examined by immunohistochemistry.

Correspondence to: Professor Mohamed Mabruk, PAPRSB Institute of Health Sciences, University of Brunei Darussalam Jalan Tungku Link, Gadong BE1410 Bandar Seri Begawan, Brunei Darussalam. Tel: +673 2463001 ext.: 2229 (Office), Fax: +673 2461502, mohamed. mabruk@ubd.edu.bn/mmabruk03@yahoo.co.uk

Key Words: Allelic imbalance/loss of heterozygosity, APC, cutaneous squamous cell carcinoma.

Materials and Methods

Samples for analysis. Twenty-two histopathologically confirmed formalin-fixed paraffin-embedded cutaneous invasive SCC were randomly selected from the archives of Pathology Department, Beaumont Hospital, Dublin, for analysis. Ethical approval was obtained from Beaumont Hospital Ethical Committee. The clinical, anatomical location and histological features of cutaneous SCC used are described in our previous report (15).

Stereomicroscopic microdissection. Hematoxylin and eosin (H&E) stained sections of each of the 22 invasive SCC were examined under light microscopy, and the areas of invasive tumor and histologically normal tissue were marked by a pathologist. A further one to three 8 μm sections (depending on the size of the tumor and the normal tissue) of each SCC sample, were then dewaxed in xylene, rehydrated through various graded alcohols, rinsed well in distilled water, and finally stained with hematoxylin. For each SCC sample, using the H&E slides as a reference, tumor and corresponding normal tissue were microdissected from the hematoxylin-stained sections under a stereomicroscope. Separate new sterile scalpel blades were used for dissecting normal and tumor tissues, and each time a new SCC sample was dissected.

For each SCC sample the corresponding histologically normal reticular dermis was used as the normal control as recommended previously(13).

Genomic DNA extraction. DNA was isolated from the microdissected tumor and normal cells using 0.5 mg/ml proteinase K (Roche Diagnostics, Mannheim, Germany) in 50 mM Tris-HCl (pH 8.5), 1 mM EDTA, 0.5% Tween) for 72 hours at 55˚C. After digestion the proteinase K was inactivated by heating to 95˚C for 10 min on a thermal cycler (Hybaid, Middlesex, UK). The tubes were then centrifuged at 12,000 ×g for 1 min. The remaining supernatant contained the genomic DNA template for subsequent PCR amplifications.

PCR detection of microsatellite marker D5S346. AI/LOH analysis of the *APC* gene region involved PCR amplification of the microsatellite marker, D5S346 (5q21-22), which is proximal to the *APC* gene. For amplification of the D5S346 marker, the forward primer was 5'-ACTCACTCTAGTGATAAATCGGG-3' and the reverse primer was 5'-AGCAGATAAGACAGTATTACTAGTT-3'. The 5' end of the forward primers also had a fluorescent CY5 label attached.

PCR amplifications were performed in a final volume of 20 μl and consisted of 1 X PCR reaction buffer, 200 μM of dNTPs, 1.5 mM of $MgCl_2$ (Promega, WI, USA), 20 pmol of each primer (MWG Biotech, UK), 2 units of *Taq* DNA polymerase (Promega) and 2 μl of sample DNA digest. PCR amplifications were carried out using an automated DNA thermal cycler (Hybaid, Middlesex, UK) and consisted of 1 cycle of denaturation at 94˚C for 5 min, followed by 1 cycle at 80˚C for 5 min, then followed by 33 cycles of denaturation for 1 min at 94˚C, annealing for 1 min at 58˚C and extension for 1 min at 72˚C, and finally by 1 cycle of elongation for 10 min at 72˚C, followed by 5 min at 25˚C.

All PCRs were carried out using a hot start with *Taq* Polymerase, *i.e.* adding the Taq once PCR reaction mixtures were at the 80˚C for 5 min step, in order to ensure optimal primer annealing (17). PCRs and subsequent fragment analysis of the D5S346 microsatellite marker were carried out in duplicate for each SCC DNA sample and its corresponding normal tissue DNA.

Following PCR, 4 μl of PCR products mixed with agarose loading dye were electrophoresed through 12% polyacrylamide mini gels. Gels were stained in a solution of ethidium bromide (100 μl of 10 mg/ml EtBr in 300 ml of dH_2O) and viewed under UV light to assess the efficiency of amplification. The intensity of the bands seen was used to determine the dilutions necessary for further analysis. The PCR product size was 100-127 bp.

Analysis of AI/LOH. The CY5 labeled PCR products (diluted in distilled water if necessary) were mixed with a CY5.5 labeled DNA size ladder and loading dye, then denatured by heating at 95˚C for 10 min and placed on ice. The samples were then analysed for AI/LOH using the OpenGene Long-Read Tower™ system DNA Sequencer (Visible Genetics, Evry, France). This involved electrophoresis of the samples in a denaturing 6% polyacrylamide gel heated to 55˚C. Normal and corresponding tumor samples were loaded side by side in the gel. The Sequencer used a laser to excite the fluorescent CY5 label on the PCR products and the CY5.5 label on the internal size markers as they moved through the gel. The Sequencer fragment analysis software was then able to accurately size the microsatellite alleles in each sample in relation to the internal size markers, and was also able to plot the measured fluorescence in terms of peak area for each microsatellite allele.

A ratio of allele peak areas was calculated for the tumor samples and their corresponding normal tissues by dividing the area under the shorter length allele peak by the area under the longer length allele peak, *i.e.* T1:T2 and N1:N2 for the tumor samples and their corresponding normal tissues, respectively (15, 18). A ratio of the ratios between the tumor and normal PCR products was then calculated to give an overall allele ratio, *i.e.* T1:T2/N1:N2 (15). In cases where the overall allele ratio calculated by this equation was greater than 1.00 the inverse was used to give a result between 0.00 and 1.00 (15, 18).

PCR amplifications and AI/LOH analysis were carried out in duplicate for each of the 22 SCC samples to ensure consistency of the results (18). An AI/LOH cut-off point of 0.88 was previously established (15). SCC samples with a mean overall allele ratio of 0.88 or less were deemed to show AI/LOH (15).

Immunohistochemical analysis of APC protein expression. Expression of the APC proteins in the 22 cutaneous SCC was examined by immunohistochemistry. Normal colon tissue was used as a positive control. Negative controls were included in which the primary antibody was omitted. Four μm sections of the formalin-fixed paraffin-embedded samples were deparaffinized, rehydrated and the antigenic sites unmasked. SCC samples were deparaffinized and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked by incubation with 0.9% H_2O_2 (BDH, UK). The antigenic sites were unmasked by pressure-cooking of the sections in 0.01 M sodium citrate solution (pH 6) for 2 min. The APC antibody used was a rabbit polyclonal antibody (sc-896; Santacruz, USA). The concentration of the primary antibody was 1/500 inTBS.

Vectastain® Universal ABC kit (Vector Laboratories,USA) was used as described by the manufacturer. For negative controls, the primary antibody was omitted. Immunostaining was visualized using 3,3'-diaminobenzidine (DAB) (Zymed,USA) as the substrate chromagen.

Table I. *LOH results at the D5S346 microsatellite marker.*

SCC sample number	Allele ratio PCR 1	Allele ratio PCR 2	Average allele ratio	LOH
1	0.84	0.72	0.78	Yes
2	0.96	0.99	0.98	No
3	0.51	0.37	0.44	Yes
4	Homozygote	Homozygote	NI	NI
5	0.93	0.95	0.94	No
6	0.99	0.94	0.97	No
7	0.26	0.28	0.27	Yes
8	0.25	0.23	0.24	Yes
9	0.92	0.91	0.92	No
10	0.40	0.35	0.38	Yes
11	0.90	0.90	0.90	No
12	0.83	0.86	0.85	Yes
13	0.18	0.16	0.17	Yes
14	0.87	0.88	0.88	Yes
15	0.95	0.90	0.93	No
16	Homozygote	Homozygote	NI	NI
17	0.58	0.41	0.50	Yes
18	0.61	0.62	0.62	Yes
19	0.96	0.95	0.96	No
20	0.92	0.92	0.92	No
21	0.85	0.67	0.76	Yes
22	0.42	0.49	0.46	Yes

NI, Non-informative.

The APC immunostaining was assessed independently by a pathologist and a scientist. For this particular APC antibody, assessment of staining was carried out according to the system used by Grace *et al*. (19). With regards to staining intensity, staining was recorded as either present or absent. Presence of staining was not rated according to the intensity of staining. Extent of staining was graded as: 0, 0-10% of cells positive; 1, 10-50% of cells positive; 2, greater than 50% of cells positive for APC.

Staining was considered positive if the extent of staining was graded as 2. Staining was considered reduced if the extent was graded as 1 and graded as 0.

Results

LOH at the D5S346 locus in the cutaneous SCC samples. Two out of 22 samples were homozygous at the D5S346 locus and therefore non-informative. Hence the rate of informativity at the D5S346 locus was 91% (20/22 samples).

The LOH results at the D5S346 locus, based on the average normal/tumor ratio for the SCC samples, obtained following fragment analysis of duplicate PCR products are shown in Table I. LOH was determined by applying the previously established cut-off point of 0.88. At the D5S346 locus, 12 (60%) of the 20 informative samples displayed LOH, with 7 (58%) of these displaying a degree of allelic loss of 50% or greater (Table I).

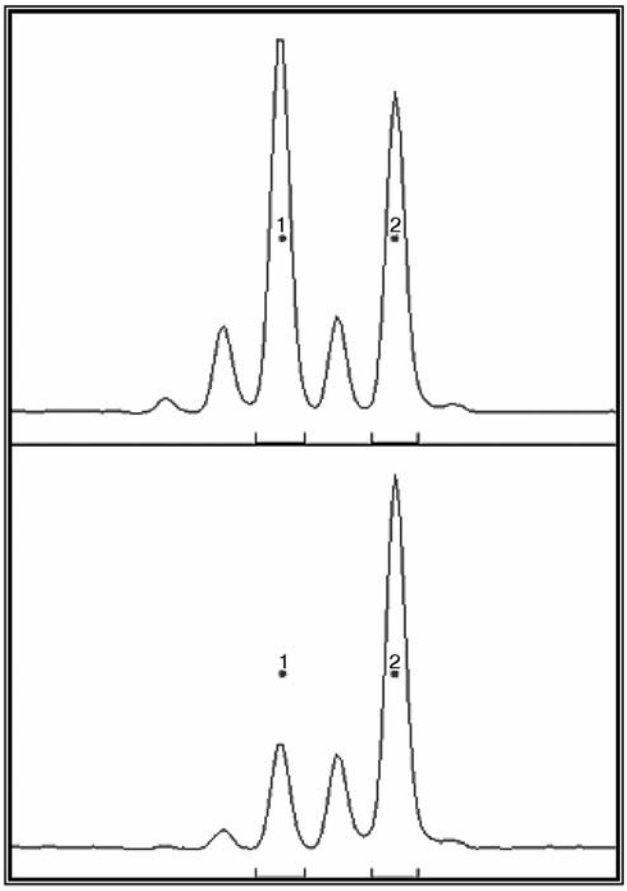

Figure 1. *Sample electropherogram generated on the Visible Genetics OpenGene Long-Read Tower System, using the fragment analysis software package, depicting the alleles at the D5S346 locus in an SCC sample and its corresponding normal tissue.*

An example of the electropherogram pattern generated when samples display retention of alleles is shown in Figure 1.

IHC expression of the APC tumor suppressor protein in the cutaneous SCC samples. The normal colon used as a positive control for this experiment displayed the expected cytoplasmic staining for APC (Figure 2). The cytoplasmic APC was concentrated in the basolateral portion of the crypt epithelial cells, with staining increasing as epithelial cells progressed from the base of the crypt to the luminal surface, suggesting an association between APC expression and maturation of colonic epithelial cells (4). However, surprisingly, none of the 22 cutaneous SCC tissue samples displayed cytoplasmic staining for APC and neither did their adjacent normal skin. Instead, it was found that 95% of SCC samples (21/22) showed nuclear staining for APC. Of these 21 samples, 13 (62%) were considered positive for nuclear APC, *i.e*. greater than 50% of cells were stained (Figure 3)

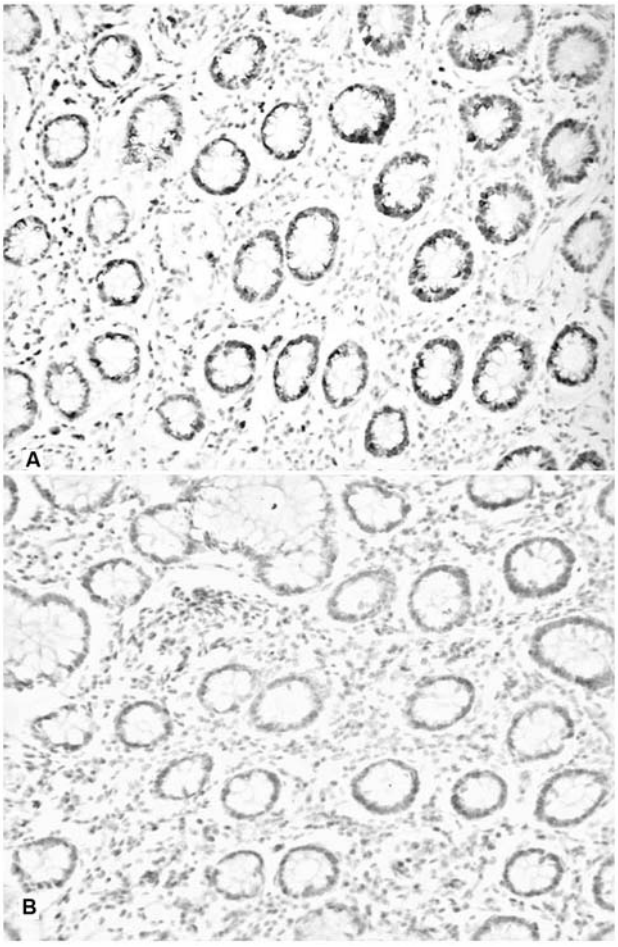

Figure 2. *A: Immunohistochemical staining of APC shows cytoplasmic expression of APC protein in normal colon tissue (magnification ×100). B: Negative control of the above tissue (magnification ×100).*

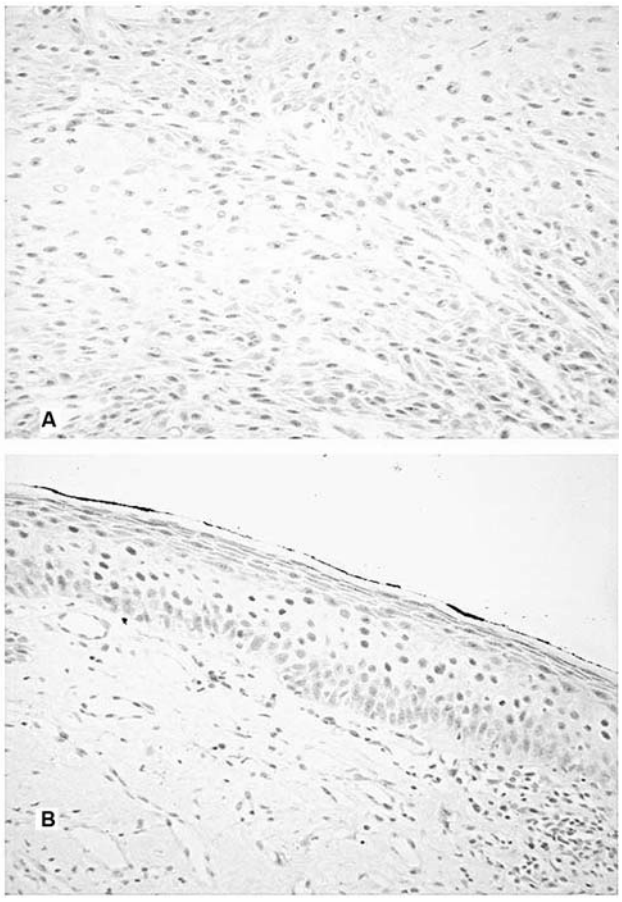

Figure 3. *A: Immunohistochemical staining of APC shows reduced nuclear expression of APC protein in the squamous cell carcinoma tumor cells of sample no. 20 (magnification ×200). B: Immunohistochemical staining of APC shows nuclear expression of APC protein in the adjacent normal epidermis of sample no. 20 (magnification ×200).*

and 8 cases (36%) showed reduced expression of APC, *i.e.* 10-49% of cells staining (Figure 3). The adjacent normal skin of 18 SCC samples also displayed some degree of nuclear APC staining, 7 samples had normal adjacent skin that was positive for nuclear APC, and 11 SCC samples had normal adjacent skin that showed reduced expression of APC. One SCC sample was negative for nuclear APC expression in both the tumor and the adjacent normal skin. Seven SCC samples were positive for nuclear APC expression in both the tumor and the adjacent normal skin (Table II).

The relationship between LOH at the D5S346 microsatellite marker, which is proximal to the *APC* locus, and expression of APC in the cutaneous SCC samples is shown in Table II.

Of the twelve SCC samples displaying LOH at the D5S346 locus, 7 samples (58%) expressed nuclear APC in the tumor, 4 (33%) showed reduced nuclear expression in the tumor and 1 sample (8%) was negative for nuclear APC expression in the tumor (Table II).

Of the eight SCC samples that did not show LOH at the D5S346 locus, 5 SCC samples (62%) were positive for nuclear APC expression and 3samples (38%) showed reduced nuclear APC expression (Table II).

Two SCC samples were non-informative at the D5S346 locus, one of these samples was positive for nuclear APC expression, the other had reduced nuclear APC expression (Table II).

Discussion

In the present study, we have used a cut-off point of 0.88. The reason for using this cut-off point is based on the fact that the cut-off point of 0.50 first used by Cawkwell *et al.* 1993 (18), and commonly referenced was chosen on the

Table II. *Summary of APC expression in the cutaneous SCC and their adjacent normal skins.*

SCC sample number	APC cytoplasmic staining		APC nuclear staining	
	Adjacent normal skin	SCC	Adjacent normal skin	SCC
1	ND	–	ND	+
2	–	–	+	+
3	–	–	Reduced	+
4	–	–	+	+
5	–	–	Reduced	+
6	–	–	–	+
7	–	–	–	–
8	–	–	Reduced	Reduced
9	–	–	+	+
10	–	–	Reduced	+
11	–	–	+	+
12	–	–	Reduced	Reduced
13	–	–	+	+
14	–	–	+	+
15	–	–	–	Reduced
16	–	–	Reduced	Reduced
17	–	–	+	+
18	–	–	Reduced	+
19	–	–	Reduced	Reduced
20	–	–	Reduced	Reduced
21	–	–	Reduced	Reduced
22	–	–	Reduced	Reduced

– , No cells staining; reduced=10-49% of cells staining; + >50% of cells staining; ND, not determined.

basis that they estimated the samples they were analysing to contain between 50-80% tumor cells and, therefore, total allelic loss in the samples containing an estimated 50% tumor cells would only give an allele ratio of 0.50 (15). However, in theory, the cut-off point of 0.50 does not apply to DNA extracted from microdissected tissue, as microdissection attempts to minimize contamination with normal cell DNA (15). In practice, however, there will undoubtedly be contamination by infiltrating inflammatory cells, and other confounding factors such as tumor heterogeneity, sensitivity of detection methods, and interpretation may artificially reduce the degree of LOH observed (15), so that complete loss of one allele, *i.e.* 100% or an allele ratio of 0.00 is rarely observed (15).

We have tried to experimentally establish a cut-off point that reflects these confounding factors and which is more appropriate for use on microdissected tissue (15). This experimentally established cut-off is more appropriate for analysis of AI/LOH in the *APC* gene in cutaneous SCC in the present study (15).

The D5S346 marker used in the present study is reported to be proximal to the *APC* tumor suppressor gene. The wild-type *APC* gene (chromosome 5q21) encodes a large protein consisting of 2,843 amino acids and having a mass of ~300 kDa (15, 19). The APC protein contains nuclear localization and nuclear export signals, which allow it (with the help of import and export receptors) to shuttle between the nucleus and the cytoplasm (20-23). The tumor suppressor activity of the APC protein has been attributed to its ability to negatively regulate β-catenin (24-25). β-Catenin plays a role in E-cadherin-mediated epithelial cell adhesion and also plays a role in the Wnt signal transduction pathway. In the presence of a Wnt signal, β-catenin accumulates in the cytoplasm, then translocates to the nucleus and coordinates with T-cell factor/lymphoid enhancer factor to activate the transcription of oncogenes such as MYC, cyclin D1 and *PPARδ* (4). The APC protein negatively regulates β-catenin activity by sequestering nuclear β-catenin and facilitates its nuclear export (20-25). Once in the cytoplasm, the APC–β-catenin complex binds with axin, glycogen synthase kinase 3β and many other proteins to form a multiprotein structure called the β-catenin destruction complex, which triggers the proteasomal degradation of β-catenin (20-25). Apart from its role in β-catenin regulation, the APC protein has also been found to associate with microtubules, suggesting that APC may also play a role in microtubule assembly (26-27).

The subcellular localization of the APC protein is reported to be predominantly cytoplasmic in normal tissues, though mammary epithelium has been reported to show equal distribution of cytoplasmic and nuclear APC (19). Midgley *et al*. have reported, however, only cytoplasmic staining for APC in normal epidermis, with the basal layer being mainly negative for APC expression (20). While the positive control of normal colon used for IHC for APC in the present study showed the expected cytoplasmic staining, all the cutaneous SCC samples analyzed were negative for cytoplasmic APC, both in the tumor cells and in the adjacent normal epidermal cells. Interestingly, however, nuclear staining for APC was found in the adjacent histologically normal skin of all but three SCC samples and was found to be expressed in the tumor cells of all but one sample of cutaneous SCC.

Olmeda *et al*. have reported that in immortalized mouse epidermal keratinocytes and in mouse cutaneous SCC cells that the cellular localization of normal APC protein depends on cell cycle stage and that there is nuclear accumulation of APC from middle S to G_2 stage of the cell cycle (28). Strong nuclear staining for APC has also been detected in alveolar soft part sarcoma(29). In a colorectal cancer cell line in which APC was found to be truncated, expression of APC was localized in the cytoplasm only. In proliferating normal canine kidney epithelial cells APC localization is predominantly nuclear, however in quiescent cells of the same type, APC expression is predominantly cytoplasmic (20).

Thus these findings suggest that normal APC protein is cytoplasmic in quiescent, terminally differentiated cells, but nuclear in proliferating cells. Although nuclear APC was expressed in the cutaneous SCC tissue samples, the fact that nuclear and not the expected cytoplasmic APC was expressed in the adjacent normal epidermal cells of the SCC samples suggests that the expressed APC is not normal. Although the adjacent normal epidermal cells of the SCC samples are histologically normal, it is possible that they have undergone genetic changes that have disrupted normal APC expression. The presence of nuclear APC staining in adjacent normal epidermis could be contributing to a field effect in carcinogenesis. The field effect could have resulted from epigenetic alterations, which include hypermethylation of the DNA promoter or could be due to genetic alterations that occur in stepwise fashion (initiation, promotion and progression (30).

Most of the *APC* mutations which have been found in colorectal cancer have been found to be frame-shift mutations, which result in a truncated protein devoid of a carboxy-terminal peptide sequence (19). This type of mutation is unlikely to be the case in the cutaneous SCCs as the antibody used was directed against the carboxy-terminus. Another study by Neufeld *et al*. has shown that mutation of both of the nuclear export signals of APC protein results in nuclear accumulation of the protein (22, 23). However, what might support our theory is a previous report published by Proweller *et al*. which showed that one mouse model of SCC does express nuclear β-catenin (31).

It is unclear as to whether or not Midgley *et al*. examined more than one normal epidermis for APC expression (20). Therefore in order to determine if the nuclear APC expression observed in the present study is abnormal, analysis of other normal epidermis from non sun-exposed sites of cutaneous SCC patients, as well as analysis of APC expression in age-matched normal epidermis of non skin cancer patients is required. Analysis of nuclear export signals APC is also necessary to determine if the nuclear protein observed is unable to export β-catenin from the nucleus and thus no longer has tumor suppressor activity.

Although 60% of the cutaneous SCCs in the present study displayed LOH at the D5S346 marker (which is proximal to the *APC* gene), there does not appear to be a correlation between LOH and the absence of APC expression. Of the samples with LOH at the APC marker, only one was negative for APC expression and three showed reduced expression, however, seven were positive for APC expression. Of the samples with no LOH, five were positive for APC expression and three samples showed reduced expression.

As it is unclear as to whether or not the expressed APC is mutated or wild-type, it is also unclear as to what the impact of LOH is at the D5S346 marker on the development of cutaneous SCC. If the expressed APC was wild-type then the LOH observed in the seven samples positive for APC expression represents a 'first hit' and is most likely to be a result of tumor development rather than a cause of tumor development. If, however, the expressed APC is mutated, then the LOH may represent a 'second hit' in which the remaining wild-type allele has been deleted, and thus may have contributed to tumor development. One SCC sample was negative for APC expression, therefore LOH may have caused deletion of one copy of the *APC* gene, and the other copy may have been inactivated by other means such as promoter methylation.

In contrast to our finding that 60% of the cutaneous SCCs displayed LOH, a previous study published by Ichihashi and Kitajima did not find LOH of the *APC* gene in cutaneous SCCs (32). The reason for this discrepancy could be due to the fact that Ichihashi and Kitajima looked at a smaller number of samples (only 5 cutaneous SCC tissue samples whereas in the present study, we have looked at 22 cutaneous SCC tissue samples).

As the APC expression patterns were similar in the SCC samples that displayed LOH and those that did not, there also remains the possibility that LOH at the D5S346 marker represents a breakpoint deletion that does not encompass the *APC* gene. Therefore examination of LOH at additional markers within the *APC* gene is needed to clarify this.

In summary, this preliminary study indicates that the *APC* gene may contribute to the genesis of cutaneous SCC. Further studies are needed in order to explore the exact role played by *APC* in the genesis of this type of malignancies.

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