ORIGINAL ARTICLE

Expression and heterodimer-binding activity of Ku70 and Ku80 in human non-melanoma skin cancer

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J Clin Pathol 2006;**59**:1181–1185. doi: 10.1136/jcp.2005.031088

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Accepted for publication 25 October 2005
Published Online First 23 February 2006

Background: Experimental data suggest that exposure to ultraviolet radiation may indirectly induce DNA double-strand breaks.
Aim: To investigate the contribution of the non-homologous end-joining repair pathway in basal and squamous cell carcinomas.
Methods: Levels of Ku70 and Ku80 proteins were determined by immunohistochemical analysis and Ku70–Ku80 heterodimer-binding activity by electrophoretic mobility shift assay. Matched pathological normal margins and skin from healthy people were used as controls.
Results: A significant increase in Ku70 and Ku80 protein levels was found for both tumour types as compared with normal skin ($p<0.001$). Squamous cell carcinoma showed increased immunostaining as compared with basal cell tumours ($p<0.02$). A direct correlation was found between Ku70 and Ku80 protein levels and expression of the proliferation markers Ki-67/MIB-1 ($p<0.02$ and $p<0.002$, respectively) in basal cell carcinoma. DNA binding activity was increased in basal cell carcinoma samples as compared with matched skin histopathologically negative for cancer ($p<0.006$). In squamous cell carcinomas, however, the difference was significant only with normal skin ($p<0.02$) and not with matched pathologically normal margins.
Conclusions: Overall, an up regulation of the Ku70 and Ku80 protein levels seems to correlate only with tumour proliferation rate. As non-homologous end joining is an error-prone mechanism, its up regulation may ultimately increase genomic instability, contributing to tumour progression.

Non-melanoma skin cancers (NMSCs), including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), are the most common skin cancers in the white population and their incidence has increased in the past 30 years. A wide range of epidemiological data supports the role of ultraviolet radiation in human skin carcinogenesis. Exposure to ultraviolet radiation leads to the formation of DNA photoproducts that are removed and repaired by the nucleotide excision repair system.[1] In the inherited disorder xeroderma pigmentosum, the failure to repair photoproducts induced by ultraviolet radiation and associated with a high incidence of skin cancers suggests an important role for DNA repair competence in the aetiology of these tumour types.[2] Besides this well-established mechanism of DNA damage, it was recently proposed that exposure to ultraviolet radiation may also cause DNA double-strand breaks (DSBs).[3] This type of molecular lesions is considered to be one of the most severe as there are difficulties in their repair that can lead to chromosomal rearrangements.[4] Exposure to ultraviolet B radiation cannot be directly responsible for the formation of DSBs; it has been suggested, however, that the photoproducts induced by ultraviolet radiation can block DNA replication, a situation favourable to DNA recombination with the formation of DSBs.[3] On the other hand, absorption of ultraviolet A radiation by cellular chromophores results in the formation of reactive oxygen species that can cause oxidative DNA damage, leading ultimately to single-strand breaks and DSBs.[5]

The cellular pathways associated with repair of the DNA DSBs are non-homologous end-joining (NHEJ) and homologous recombination pathways.[4] The key element of the NHEJ pathway is the Ku70–Ku80 heterodimer, which binds the DNA and recruits the DNA protein kinase catalytic subunits to the site; the DNA ligase 4 and XRCC4 intervene in the final ligation step.[4] This mechanism of DNA repair, however, is non-conservative, because it may lead to erroneous rejoining of the broken ends, causing a loss or amplification of chromosomal material, and even translocation.[6] The homologous recombination pathway accounts for 30–50% of endonuclease-induced DSB repair events in mammalian cells, and it is more conservative than NHEJ. But some of the components of the DSB repair pathway, such as the sensor complex RAD50–MRE11–NBS1, are associated with both NHEJ and homologous recombination, suggesting that the two mechanisms are distinct but complementary processes.[6]

We have previously reported a modulation of protein expression and Ku70–Ku80 binding activity, during tumour progression in a patient with multiple basal cell carcinomas.[7] In this study, we analysed protein expression and Ku70–Ku80 DNA-binding activity in a series of BCCs and SCCs. We found a substantial increase in protein expression in neoplastic samples as compared with normal controls without cancer. Significantly higher levels of protein expression were detected in SCC than in BCC, and a significant correlation with the proliferation marker Ki-67 was found in BCC ($p<0.02$). Binding activity was also increased in BCC as compared with matched pathological normal margins.

MATERIALS AND METHODS

Patients and samples

The institutional review board approved the study.

Abbreviations: BCC, basal cell carcinoma; DSB, double-strand break; EMSA, electrophoretic mobility shift assay; IHC, immunohistochemical; NHEJ, non-homologous end joining; NMSC, non-melanoma skin cancer; SCC, squamous cell carcinoma; TBS, TRIS-buffered saline

Samples of the tumour and matched histopathological normal skin from margins from 20 patients with BCCs and 8 patients with SCCs were obtained from the Plastic Surgery Division, Campus Bio-Medico University, Rome, and the Department of Dermatology, Federico II University, Naples. On the basis of the histopathological growth patterns, BCC can be classified into four subtypes: superficial, nodular and micronodular, infiltrative or morphoeic, and mixed.[8] According to Rippey[8] and Staibano *et al*,[9] superficial and nodular BCCs were considered to be non-aggressive types and grouped as BCC1, and micronodular and infiltrative or morphoeic BCCs were considered to be aggressive types and grouped as BCC2. The patients' skin types were II and III and none of them had received radiotherapy before surgery. Normal skin samples from healthy people (n = 5) were analysed as controls. All specimens were obtained from patients who had not been exposed to *x* rays or to chemical carcinogens, known as DNA DSB repair-inducing agents.[10 11] Seventy five per cent of the tumours were localised on sun-exposed areas; occupational exposure to ultraviolet radiation was reported in the remaining cases. As controls, normal skin samples from sun-exposed areas obtained from five healthy people without occupational exposure to ultraviolet radiation were analysed.

Immunohistochemical analysis

Owing to tumour size, immunohistochemical (IHC) analysis could be carried out only on paraffin-wax-embedded slides from 16 BCCs and 6 SCCs. On the basis of an initial review of all available slides of the surgical specimen sections stained with haematoxylin and eosin, we selected one representative block of paraffin wax from each case for further study. Consecutive 3-μm sections were cut from each block and were immunostained for p53, Ki-67/MIB-1, bcl-2, Ku80 and Ku70. IHC staining was carried out by the streptoavidin–biotin method. Briefly, sections were deparaffinised and microwave-treated at 500 W for 5 min twice in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase was blocked by incubating slides in 0.03% hydrogen peroxide in absolute methanol for 30 min at room temperature. The antibodies used were monoclonal mouse antibodies against human p53 protein (clone DO7; Dako, Glostrup, Denmark; 1:50), human bcl-2 protein (clone 124; Dako; 1:40) and anti-Ki-67 protein (clone MIB-1; Dako; 1:50), goat polyclonal antibodies against Ku70 and Ku80 proteins (M-19, M-20; Santa Cruz Biotechnology, Santa Cruz, California, USA; 1:200). All primary antibodies were incubated at room temperature for 2 h. After washing three times with TRIS-buffered saline (TBS), sections treated with anti-p53, anti-bcl-2 and anti-Ki-67 were incubated with biotinylated goat antimouse or antirabbit immunoglobulin G and sections treated with anti-Ku70 and anti-Ku80 were incubated with biotinylated swine antigoat, antimouse or antirabbit immunoglobulin G (Dako). They were then washed with TBS, treated with streptoavidin–peroxidase reagent (Dako) for 10 min and washed three times with TBS again. Finally, specimens were incubated in diaminobenzidine for 5 min, followed by haematoxylin counterstaining. Negative control slides, processed without primary antibody, were included for each staining run.

IHC staining with anti-Ku70, anti-Ku80, anti-p53 and anti-Ki-67 antibodies showed nuclear localisation, whereas staining of tumours with anti-bcl-2 antibody showed a cytoplasmic localisation for bcl-2 protein. Each IHC analysis was carried out independently and reactivity was assessed as positive only when tumours showed intense staining. For each carcinoma, IHC staining was evaluated at the invasive edge of the tumour and at least 1000 tumour cells were observed. The staining score for Ku70, Ku80, p53 and Ki-67 was evaluated as the percentage of stained cells out of the total number of cells evaluated. bcl-2 expression was graded on a scale of 0 to 3+: 0, no cells positive; 1+, <25% positive; 2+, 26–50% positive; and 3+, >50% positive.[12 13] Two pathologists (GP and CR), unaware of clinical data and molecular results, examined sections under a two-head microscope.

Preparation of nuclear extracts and analysis of DNA end-binding activity

Immediately after surgical resection, tissue samples were processed for protein extraction, according to the Dignam method.[14] DNA binding activity was determined by electrophoretic mobility shift assay (EMSA).[15 16] A phosphorus-32-end labelled 56-bp DNA probe that is recognised by the Ku70–Ku80 heterodimer[17] was incubated with nuclear extracts for 30 min at room temperature in binding buffer (10 mM TRIS–hydrochloric acid (HCl), pH 8, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 150 mM sodium chloride (NaCl), 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 10% glycerol). The DNA-binding reactions contained the labelled probe (50 000 cpm), nuclear extracts (2 μg) and closed circular plasmid DNA pUC-19 (1 μg) as an unspecific competitor. To normalise the samples, EMSA was carried out by incubating the nuclear extracts (2 μg) with 50 000 cpm/sample of ^{32}P-end-labelled Sp-1 oligonucleotide (Promega Corporation, Madison, Wisconsin, USA) in binding buffer (20% glycerol, 5 mM magnesium chloride ($MgCl_2$), 2.5 mM EDTA, 250 mM NaCl, 50 mM TRIS–HCl, pH 7.5, 2.5 mM dithiothreitol) with 1 μg of poly[dI-dC] as an unspecific competitor. For gel supershift experiments, goat polyclonal anti-Ku70 and anti-Ku80 antibodies (Santa Cruz Biotechnology) were incubated with protein extracts for 30 min at room temperature before adding the other components of the binding reaction. Complexes were separated on 6% non-denaturing polyacrylamide gels in TRIS–borate–EDTA, pH 8.0 at 200 V for 2 h and 30 min, respectively. Gels were dried and exposed to *x* rays (Amersham Pharmacia Biotech, Piscataway, USA) overnight at −80°C. The optical densities were obtained by scanning densitometry, with colon carcinoma cell line extracts (CaCo-2, American Type Culture Collection) as internal control (optical density 5.41 (0.71)).

Statistical analysis

Data were statistically analysed with SPSS V.10.1. Non-parametric tests were used to analyse continuous variables. Wilcoxon's test was used to compare data within groups, and the Mann–Whitney U test to compare data between groups. Changes in distribution of categorical variables were determined with the χ^2 test. The correlation between Ku70 or Ku80 expression and Ki-67/MIB-1 was determined using Pearson's correlation test. Differences were considered significant at $p<0.05$.

RESULTS

Up regulation of Ku70 and Ku80 in NMSC

BCC (n = 16) and SCC (n = 6) samples were stained with antibodies anti-Ku70 and anti-Ku80. Normal skin samples from healthy people (n = 5) were also used as controls (fig 1A). Both BCC and SCC samples showed a significant increase in expression levels of Ku70 and Ku80 as compared with controls ($p<0.006$; table 1), as well as a significant increase in Ku70 and Ku80 immunostaining when SCC and BCC were compared ($p<0.02$; table 1). Interestingly, a direct correlation was found between Ku70 and Ku80 immunostaining and Ki-67/MIB-1 expression in BCC ($p<0.02$ and $p<0.002$, respectively; fig 2).

EMSAs were carried out on nuclear extracts from the 5 normal skin samples from healthy people, 16 from BCCs and

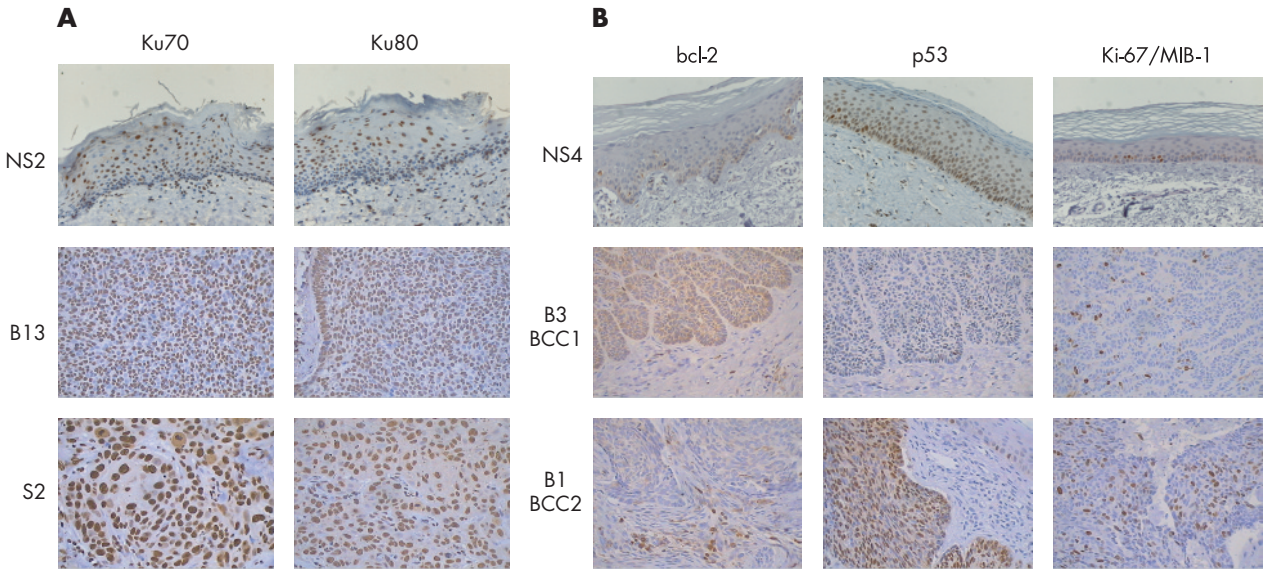

Figure 1 Protein expression in non-melanoma skin cancer. Representative results of paraffin-wax-embedded sections stained with the specific antibody. (A) Nuclear expression of Ku70 and Ku80 proteins in normal skin (NS2), basal cell carcinoma (BCC; B13) and squamous cell carcinoma (SCC; S2). (B) Protein immunostaining for bcl-2, p53 and Ki-67/MIB-1 in normal skin (NS4), non-aggressive (B3) and aggressive BCCs (B1). The percentage of stained cells for p53 and Ki-67/MIB-1 is higher in B1 than in B3, whereas B3 shows 3+ staining for bcl-2, and B1 shows 1+ staining.

6 from SCCs. Four additional BCCs for which immunostaining could not be carried out were also tested. For two SCC samples and one BCC sample tested by immunohistochemical analysis, no fresh tissue was available for nuclear extraction. Figure 3A shows the representative results. In pathologically normal margins from patients with BCC and SCC, the activity was increased as compared with normal skin from healthy people ($p<0.02$). An increase in DNA-binding activity was shown in BCC as compared with normal skin ($p<0$) and matched pathologically normal margins ($p<0.006$). For SCC, the difference was significant with normal skin ($p<0.02$) but not with matched pathologically normal margins (fig 3B).

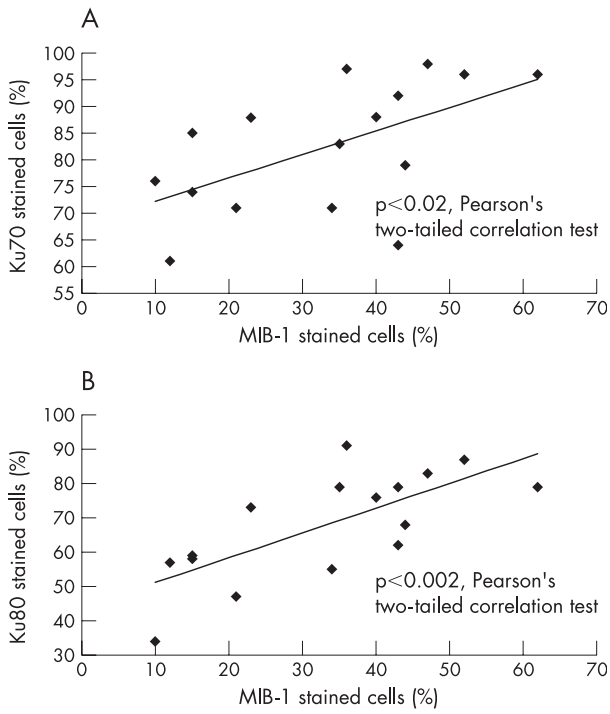

Figure 2 Correlation between Ki-67/MIB-1 and immunostaining of Ku70 and Ku80 proteins in basal cell carcinoma. A statistically significant direct correlation was found for both Ku70 (A) and Ku80 (B).

Markers of progression in BCCs

BCC samples were classified as non-aggressive (BCC1) and aggressive (BCC2) on the basis of the histological growth pattern,[8] and expression of bcl-2, and Ki-67/MIB-1 markers[18–20] (fig 1B). As expected, bcl-2 immunostaining was more common in BCC1 ($n = 9$) than in BCC2 ($n = 7$; $p<0.05$), and expression of Ki-67/MIB-1 was 50% lower in BCC1 than in BCC2 tumours ($p<0.02$). Expression of p53 did not show a marked increase in the two BCC subtypes. Analysis of Ku70 and Ku80 protein expression showed a slight, although not statistically significant, increase of immunostaining in BCC2 as compared with BCC1 (table 1), but no changes were detected for DNA-binding activity (fig 3b).

DISCUSSION

To determine whether changes in the NHEJ pathway activity are associated with NMSC carcinogenesis, we determined Ku70 and Ku80 protein levels and binding activity of Ku70–Ku80 in a series of BCCs and SCCs. As increased expression of Ki-67/MIB-1, p53 and bcl-2 markers were previously correlated with tumour aggressiveness in NMSC, their level was also determined by immunohistochemical analysis.

Immunostaining analysis showed a statistically significant increase in protein expression of Ku70 and Ku80 in BCC and SCC as compared with normal skin from healthy people, and a substantial increase in SCC as compared with BCC. Both proteins show the same behaviour as Ki-67/MIB-1 and bcl-2, which are known markers of aggressive behaviour in NMSC. Moreover, a direct correlation was found in BCC between immunostaining of Ku proteins and MIB-1 expression, suggesting that Ku70 and Ku80 expression may be related to an increased proliferation rate. The binding activity was already increased in histopathologically normal margins from the patients as compared with skin from healthy people and was further increased in BCCs but not in SCCs. These results suggest that the NHEJ pathway may be activated in the

Table 1 Expression levels of proteins in normal skin and non-melanoma skin cancer

Samples	n	Ku70*	Ku80†	Ki-67/MIB-1‡	p53§	bcl-2¶		
						0	1–2	3
Normal skin	5	62 (4)	42 (3)	ND	ND		ND	
BCC tot	16	82 (3)	68 (4)	33 (4)	53 (5)	2	6	8
BCC1	9	80 (4)	63 (6)	25 (5)	45 (9)	1	1	7
BCC2	7	85 (5)	74 (5)	44 (3)	60 (6)	1	5	1
SCC	6	94 (2)	87 (3)	62 (2)	74 (5)	6	0	0

Results for Ku70, Ku80, Ki-67/MIB-1 and p53 are expressed as mean (SEM) percentage of positively stained cells; for bcl-2, numbers indicate the intensity of staining.[12 13]
BCC, basal cell carcinoma; ND, not determined; SCC, squamous cell carcinoma.
*Normal skin *v* BCC tot, $p<0.006$; normal skin *v* SCC, $p<0.004$; BCC tot *v* SCC, $p<0.021$; Mann–Whitney test.
†Normal skin *v* BCC tot, $p<0.003$; normal skin *v* SCC, $p<0.004$; BCC tot *v* SCC, $p<0.005$; Mann–Whitney test.
‡BCC tot *v* SCC, $p<0.000$; BCC1 *v* BCC2, $p<0.016$; Mann–Whitney test.
§BCC tot *v* SCC, $p=0.049$; Mann–Whitney test.
¶BCC tot *v* SCC, $p=0.000$; BCC1 *v* BCC2, $p<0.05$; χ^2 test.

attempt to prevent or reduce the development of genetic instability.

According to the histopathological growth patterns, BCC can be divided into four subtypes: superficial, nodular (including micronodular), infiltrative or morphoeic and mixed.[8] Superficial and nodular BCCs express high levels of bcl-2 and low levels of proliferation-associated Ki-67 antigen (BCC1), suggesting that extended cell survival plays a more important part than cell proliferation in their development. On the contrary, the infiltrative or morphoeic and micronodular type of BCCs[8] express lower levels of bcl-2 and high levels of Ki-67 antigen (BCC2). In a patient with multiple basal cell carcinomas, we have previously reported an increase in Ku70 and Ku80 protein expression levels and DNA-binding activity in tumours showing a non-aggressive pattern, and a marked decrease in DNA-binding activity in the aggressive tumours.[7] In our study, however, only a slight reduction of DNA-binding activity was found in aggressive tumours (BCC2) as compared with the non-aggressive tumours (BCC1; fig 3).

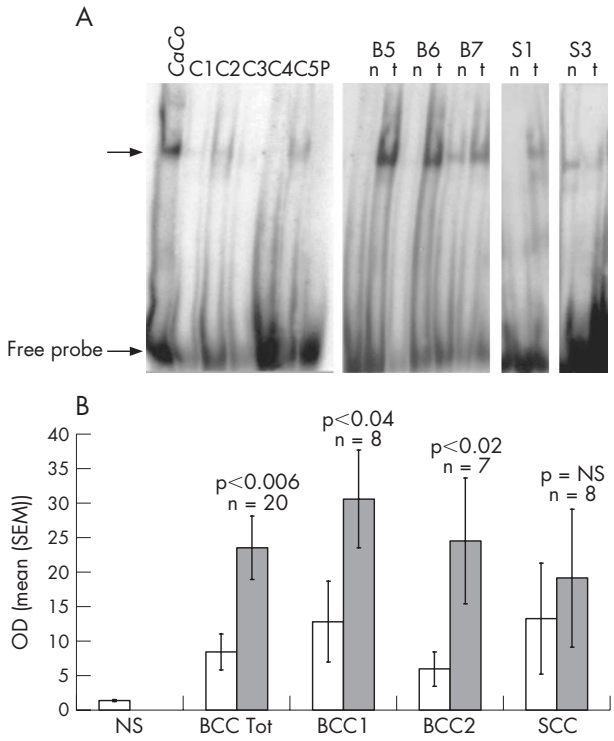

Figure 3 DNA-binding activity of Ku70–Ku80 in non-melanoma skin cancer. (A) Representative results of electrophoretic mobility shift assay (EMSA) of a protein–DNA complex. Arrows indicate free probe and specific protein–DNA complexes. From left to right, CaCo, CaCo2 cell line protein extract (HTB 37, American Type Culture Collection); C1–C5, normal skin samples from healthy people; P, free probe without protein extract; B5–B7, basal cell carcinomas; S1, S3, squamous cell carcinomas; n, nuclear extract from pathologically normal margin; t, nuclear extract from tumour specimen. (B) DNA-binding activity of Ku70–Ku80, histograms are mean (SEM) values of optical densities (OD) obtained by scanning densitometry of band shifts. NS, normal skin from healthy people; BCC, total basal cell carcinoma; BCC1, non-aggressive basal cell carcinoma; BCC2, aggressive basal cell carcinoma; SCC, squamous cell carcinoma. White bars, normal; black bars, tumour. p Values were determined as described in Materials and methods.

A "caretaker" role has been proposed for the NHEJ pathway,[6 21 22] and the down regulation of the system was reported in more advanced and metastatic malignancies as compared with benign lesions or less aggressive tumours.[23 24] Other studies, however, indicate that NHEJ itself may cause chromosomal rearrangements.[25 26] The data presented here support the hypothesis that the NHEJ pathway is substantially activated in NMSC. This activation may be a response to the failure of other repair mechanisms such as the nucleotide excision repair system that plays such an important part in defending skin from solar radiation. On the other hand, as suggested by the correlation with MIB-1, the increase in Ku70 and Ku86 protein expression may be related to high proliferation rates that enhance the likelihood of DNA breaks requiring a more reactive DNA repair system.[27] Our data suggest a substantial up regulation of the key element of the NHEJ pathway in NMSC that seems mainly correlated with tumour cell proliferation rate. As the NHEJ system is an error-prone mechanism,[25 26] this up regulation could increase the genotoxic effect of solar radiation, ultimately contributing to the progression of skin cancer.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministero della Salute (Italian Ministry of Health; IRCCS RC2004 and RC2005), from MIUR (Italian Ministry of Instruction, University and Scientific and Technological Research; COFIN 2002) and from the PhD programmes from the Campus Bio-Medico University, Rome, and Federico II University, Naples.

Take-home message

- The non-homologous-end joining pathway, a critical mechanism for resolving DNA double-strand breaks, is associated with non-melanoma skin cancer progression.

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Competing interests: None declared.

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