Retinoic Acid Increases the Expression of p53 and Proapoptotic Caspases and Sensitizes Keratinocytes to Apoptosis: A Possible Explanation for Tumor Preventive Action of Retinoids

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ABSTRACT

Retinoids influence growth and differentiation of keratinocytes (KCs) and are widely used for the management of skin diseases and for prevention of nonmelanoma skin cancer (NMSC) in predisposed patients. Here we investigated the effect of all-trans-retinoic acid (ATRA) on KC apoptosis. When KCs were cultured in confluent monolayers for several days, they acquired resistance against UVB-induced apoptosis. In contrast, when the cells were treated with 1 μmol/L ATRA for 6 days and subsequently irradiated with different doses of UVB, they underwent massive apoptosis as assessed by morphology, expression of activated caspase-3, and DNA fragmentation. The same effect was observed when doxorubicin was used instead of UVB. Analysis by real-time PCR and Western blot revealed that ATRA treatment strongly increased the mRNA and protein expression of p53 and caspase-3, -6, -7, and -9, which are key regulators of apoptosis. UVB irradiation of ATRA-treated cells but not of control cells led to the accumulation of p53 protein and of its target gene Noxa. Inhibition of p53 and caspases with α-pifithrin and z-Val-Ala-Asp-fluoromethyl ketone, respectively, blocked UVB- and doxorubicin-induced apoptosis in ATRA-treated KCs. Analogous to the observed ATRA effects in monolayer cultures, in vitro-generated organotypic skin cultures reacted with up-regulation of p53 and proapoptotic caspases and displays increased sensitivity to UVB-induced apoptosis. The ability of retinoic acid to regulate the expression of proapoptotic genes and to sensitize KCs to apoptosis may play a role in their prevention of NMSC in transplant patients and patients with DNA-repair deficiencies.

INTRODUCTION

Retinoids are powerful modulators of cell proliferation, differentiation, and apoptosis and a mainstay therapy for a wide range of dermatologic diseases (1). In addition to their use in psoriasis and acne therapy (2), retinoids effectively prevent the development of nonmelanoma skin cancer (NMSC) in patients with defects of DNA repair, such as xeroderma pigmentosum (3), and in transplant patients receiving immune-suppressive therapies (4). Despite their use for many years, their mode of action in the prevention of NMSC still is unclear (5). Contrary to what would be expected of an anticancer drug, retinoids affect proliferation of keratinocytes (KC)s only moderately in vitro (6) and even induce KC proliferation when applied topically in vivo (7). In contrast to KCs, leukemic cell lines and cells derived from patients with acute promyelocytic leukemia differentiate and can undergo apoptosis when exposed to retinoids (1). Apoptosis in this setting has been attributed to the induction of tumor necrosis factor-related apoptosis-inducing ligand expression by retinoids and its paracrine effect on tumor cells (1).

In normal cells, DNA damage leads to the activation of protective mechanisms (e.g., DNA repair) that prevent the accumulation of mutations and emergence of neoplastic cells. An important step in DNA repair is a cell-cycle arrest mediated by the tumor suppressor protein p53 (8). The occurrence of DNA lesions as a consequence of UVB irradiation or treatment with cytotoxic agents, such as doxorubicin, leads to post-translational modifications of p53 (9), resulting in p53 accumulation and increased activity (10) and subsequently in transcriptional activation of p21 (11) and GADD45 (12), leading to cell-cycle arrest. If DNA damage is irreparable, p53 action leads to apoptosis (8), which is, at least in part, mediated by transcriptional up-regulation of proapoptotic members of the bcl-2 family, such as Bax (13), Noxa (14), and PUMA (15). In contrast, MDM2, another p53-dependent gene, inhibits p53 activity (9), thereby establishing an autoregulatory negative feedback loop (16). Mutations of p53, as in Li Fraumeni syndrome, and deletion of p53 in experimental animals predispose to the development of neoplastic tumors (17, 18). Overexpression of p53 by itself does not induce apoptosis; rather, it lowers the apoptotic threshold in response to DNA damage (19, 20). Importantly, experimental evidence indicates that the proapoptotic activity and the chemopreventive effect of retinoids depend on the presence of wild-type p53 (21–23). However, p53 by itself is not sufficient for apoptosis induction by retinoids. The synthetic retinoid CD437 is able to induce apoptosis in human lung cancer cells only when they contain functional p53, whereas primary lung epithelial cells are not susceptible (21, 24).

Caspases are cysteine-dependent aspartic proteases that are divided into proapoptotic (caspase-2, -3, -6, -7, -8, -9, and -10) and proinflammatory (caspase-1, -4, and -5) members (25). They are expressed as inactive proenzymes that are activated by proteolytic cleavage into large and small subunits and form tetrameric complexes (25). Proapoptotic caspases are grouped into initiator (caspase-8, -9, and -10) and executor (caspase-3, -6, and -7) caspases (25). Although caspase activity is regulated primarily at the post-translational level, overexpression of caspases sensitizes cells for apoptosis (26, 27).

We recently have shown that retinoids down-regulate the epidermis-specific caspase-14, which appears not to be involved in classical apoptosis but is thought to play a role during terminal KC differentiation (28). In contrast to caspase-14, the proapoptotic caspase-3 was up-regulated, and caspase-8 expression remained unchanged, indicating differential regulation of caspases by retinoids (29). Furthermore, we observed spontaneous retinoid-induced apoptosis of KCs cultured under differentiating conditions (29). In the present report, we further investigate this observation and show that retinoids induce the expression of p53 and several proapoptotic caspases, leading to increased sensitivity to UVB- and doxorubicin-induced apoptosis.

MATERIALS AND METHODS

Antibodies and Reagents. Mouse monoclonal antibodies against the following proteins were used in the present study: caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), caspase-6 (Medical & Biological Laboratories, Nagoya, Japan), caspase-7 (NeoMarker, Fremont, CA), caspase-9 (Oncogene, Boston, MA), p53 (Immunotech, Marseille, France), and p63 (NeoMarker). A rabbit antisera directed against cleaved caspase-3 (R&D Systems, Minneap-
oligos, MN) was used to detect activated caspase-3. The pan-caspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk; Alexis, Carlsbad, CA) and the p53 inhibitor α-pifithrin (Alexis) were dissolved in dimethylsulfoxide and used at 40 μM and 90 μM, respectively. Bis-benzimide ( Hoechst 33258) was obtained from Sigma (St. Louis, MO). All-trans-retinoic acid (ATRA; Sigma) was dissolved in dimethylsulfoxide and stored in 10^−2 mol/L aliquots at −20°C protected from light.

**Cell Culture.** Human neonatal foreskin-derived KCs were obtained from Clonetics (San Diego, CA) and cultured as described previously (29). Briefly, second- to fourth-passage KCs were seeded at 80% confluence in KC growth medium containing fibroblasts. After overnight incubation, the medium was removed, replaced with PBS before UVB irradiation. After irradiation, KC basal medium containing 1% BSA (Sigma) was added daily, and the cells were treated for 6 days unless indicated otherwise.

**UVB Irradiation.** UVB irradiation was carried out with a Waldmann F15T8 15W lamp (Villingen-Schwenningen, Germany), and the medium was replaced with PBS before UVB irradiation. After irradiation, KC basal medium was added to the cultures. Z-VAD-fmk (40 μM/L) and α-pifithrin (90 μM/L) were added after UVB irradiation. In experiments with SE, these inhibitors were added 20 hours and 3 hours before UVB irradiation, and serum-free KC defined medium was changed to KC basal medium before UVB exposure. If not indicated otherwise, KCs and SEs were analyzed 24 hours after UVB irradiation.

**Immunostaining.** SEs and KCs grown on coverslips were immunostained as described previously (31). Briefly, cells that were cultured on coverslips were fixed in 1% paraformaldehyde and perforated by incubating them in PBS (0.1%) and PBS Tween (0.1%). Both cells treated this way and formalin-fixed paraffin sections of SE were finally incubated in 2% BSA (Sigma)/10% goat serum (DAKO, Glostrup, Denmark) and with primary and secondary antibodies (Alexa 546; Molecular Probes, Eugene, OR). Nuclear staining was achieved by incubating the sections with Hoechst 33258 (Sigma).

**Analysis of DNA Fragmentation.** Equal numbers of pooled, detached, and adherent KCs were centrifuged, and the pellets were incubated in 500 μL lysis buffer [5 mM Tris (pH 7.4), 0.5% Triton, and 5 mM/L EDTA] for 20 minutes on ice. After centrifugation, the supernatants containing fragmented, cellular DNA were treated with proteinase K and RNase A. The DNA was extracted and separated on an agarose gel. DNA Molecular Weight Marker VI (Roche, Basel, Switzerland) was loaded in parallel in each experiment.

**Western Blot Analysis.** For the analysis of protein expression, samples were lysed in an SDS buffer [62.5 mM/L Tris/HCl (pH 6.8), 6 mol/L urea, 2% SDS, 0.00125% bromophenol blue, and 5% β-mercapto-EtOH]. Western blot analysis was carried out as described previously (29). Briefly, after separation in a polyacrylamide gel, blotting membranes were blocked (PBS with 7.5% nonfat dry milk, 2% BSA, and 0.1% Tween) and incubated with primary and secondary peroxidase-conjugated goat antibodies (Pierce, Rockford, IL). The membranes were developed using either the ECL chemiluminescence detection system (Amersham, Piscataway, NJ) or Chemilux (Alphaphinotech, CA). For all of the experiments, Ponceau staining of filters was performed to confirm loading of equal amounts of protein.

**Real-Time PCR.** RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA), and reverse transcription was carried out with Gene Amp RNA PCR (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed by LightCycler technology (Roche Molecular Biochemicals) using a standardized program (10° denaturing step; 55 cycles of 5° at 95°C, 5° at 65°C, and 15° at 72°C; melting point analysis in 0.1°C steps; final cooling step). Each LightCycler capillary was loaded using LightCycler FastStart DNA Master SYBR Green 1 kit (Roche), with 1.5 μL DNA Master Mix, 1.8 μL MgCl2 (25 mM/L), 10.1 μL H2O, and 0.5 μL of each primer (10 μM/L). Relative quantification of target gene expression was performed using a mathematical model described by Pfaffl et al. (32). The following primers were generated (MWG Biotech, Ebersberg, Germany): caspase-3_f(5’-aggaaggcaaatggaagct-3’), caspase-3_r(5’-tacgcaacaagggcagaga-3’), caspase-6_f(5’-atctcgagaagtgctgtaattgg-3’), caspase-6_r(5’-tcggagttcctcgagagagct-3’), caspase-7_f(5’-gaggccggaagttctttgacaagct-3’), caspase-7_r(5’-agaggcacgtgcaaatggaagct-3’), caspase-9_f(5’-aatggcgagtctgctcattgg-3’), caspase-9_r(5’-tcggagttcctcgagagagct-3’), p53_f(5’-ctcggagaagtgctgtaattgg-3’), p53_r(5’-tcggagttcctcgagagagct-3’), MDM2_f(5’-tggcagttcctcgagagagct-3’), MDM2_r(5’-tcggagttcctcgagagagct-3’), Noxa_f(5’-tcggagttcctcgagagagct-3’), and Noxa_r(5’-tcggagttcctcgagagagct-3’). In all of the experiments, the results were normalized to the expression of the housekeeping gene. For the analysis of protein expression, samples were loaded in parallel with each experiment.

**Fig. 1.** ATRA sensitizes KCs to UVB-induced apoptosis. A. Untreated (a, c, e, and g) or retinoid-treated (b, d, f, and h) SEs were irradiated with 0 (a-d) or 100 mJ/cm² UVB (e-h) and analyzed by H&E (a, b, e, and f) and active caspase-3 staining (red; c, d, g, and h) or presence (c, d, g, and h) of ATRA. Sham (a-d) or UVB-irradiated (60 mJ/cm²; e-h) KCs were photographed directly (a, c, e, and g) or after staining for active caspase-3 (red) and nuclei (blue; b, d, f, and h). C. Alternatively fragmented DNA derived from KCs of the different groups was separated on an agarose gel.
gene 5-aminolevulinic acid synthase, using the following primers: alas_f (5'-ccactggaagagctgtgtga-3') and alas_r (5'-acccctcaacacaacaa-3').

RESULTS

ATRA Sensitizes KCs to UVB-Induced Apoptosis. When normal human epidermal KCs are grown in SE culture and are maintained for 6 days at the air-liquid interface, a multilayered epidermis is formed (ref. 30; Fig. 1A, a). Addition of 1 μmol/L ATRA during the 6-day differentiation period, a concentration which is achieved in the course of topical retinoid therapy (33), leads to epidermal thickening with impaired stratification and cornocyte layer formation (Fig. 1A, b) and to spontaneous apoptosis of 10 to 20% of KCs (ref. 29; Fig. 1A, d). To investigate whether KCs in ATRA-treated SEs also are less resistant to a standard proapoptotic stimulus, we irradiated SEs with UVB. In control SEs exposed to UVB, only few basal cells displayed apoptotic morphology with nuclear condensation and expression of activated caspase-3 (Fig. 1A, e and g). In contrast, a dramatic apoptotic effect on virtually all of the suprabasal KCs was observed in the ATRA-treated sample (Fig. 1A, f and h). These data show that in addition to increasing spontaneous KC apoptosis, ATRA treatment of SEs sensitizes KCs to UVB-induced apoptosis.

To study the basic mechanisms of ATRA on the apoptotic threshold observed in SEs, we went back to the simpler model of KCs grown in monolayer culture in the absence of fibroblasts. KCs grown under differentiating conditions (34) in 0.15 mmol/L calcium at confluence for several days were resistant to UVB-mediated apoptosis (Fig. 1B, e and f; ref. 35). In contrast, ATRA-treated KCs became highly apoptotic after UVB exposure as shown by morphology (Fig. 1B, g), active caspase-3 staining (Fig. 1B, h), and DNA fragmentation (Fig. 1C, Lanes 5 and 7).

Fig. 1B. ATRA increases UVB-induced apoptosis in confluent KCs. Confluent KCs were cultured in the presence or absence of 1 μmol/L ATRA for 6 days and irradiated with UVB (120 mJ/cm²). Immediately before (0 hour), and 4, 8, and 12 hours after UVB irradiation, RNA was extracted and analyzed with real-time PCR to determine the mRNA level of p53 (A) and MDM2 and Noxa (C); ∗, P < 0.05; ∗∗, P < 0.0005; ∗∗∗, P < 0.000005. B, Western blot analysis was performed to determine the protein expression levels of p53.

ATRA Inhibits Down-Regulation of p53 and Increases the Expression of Caspases in Confluent KCs. When KCs were grown for 4 days at confluence, expression of p53 mRNA (Fig. 2A) and protein (Fig. 2B) strongly decreased. However, addition of ATRA to the culture medium completely inhibited down-regulation of p53 (Fig. 2A and B). The expression of p63, a p53 family member that has antiapoptotic effects in KCs (36), also was reduced by retinoid treatment, although much less than that of p53 (Fig. 2B). The increased expression of p53 was paralleled by an induction of MDM2 (Fig. 2C) but not of the proapoptotic Noxa (Fig. 2C). These data show that ATRA treatment of KCs prevents confluence-associated down-regulation of the tumor suppressor protein p53 and leads to an increased transcription of MDM2, a p53 target gene that counterbalances p53 activity.

Next we focused on the effect of UVB irradiation on p53 expres-

Fig. 2. ATRA inhibits confluence-induced down-regulation of p53. The mRNA expression of p53 (A) and MDM2 and Noxa (C) of confluent KCs was determined with real-time PCR; ∗, P < 0.05; ∗∗, P < 0.005. B, Protein lysates obtained 2, 4, and 6 days after start of ATRA addition were analyzed by Western blot for p53 and p63.

Fig. 3. UVB irradiation leads to p53 accumulation in ATRA-treated KCs. Confluent KCs were cultured in the presence or absence of 1 μmol/L ATRA for 6 days and irradiated with UVB (120 mJ/cm²). Immediately before (0 hour), and 4, 8, and 12 hours after UVB irradiation, RNA was extracted and analyzed with real-time PCR to determine the mRNA level of p53 (A) and MDM2 and Noxa (C); ∗, P < 0.05; ∗∗, P < 0.0005; ∗∗∗, P < 0.000005. B, Western blot analysis was performed to determine the protein expression levels of p53.
sion and activity. After UV irradiation, mRNA levels for p53 mRNA remained elevated in ATRA-treated as compared with nontreated KCs but showed no further increase (Fig. 3A). In contrast, p53 protein levels strongly increased after UVB exposure of ATRA-treated but not of nontreated KCs (Fig. 3B), showing p53 protein accumulation. Simultaneously, a rapid decrease of MDM2 mRNA paralleled by a steep increase of Noxa, which was much more pronounced in ATRA-treated cells, was observed (Fig. 3C).

Next we analyzed caspase-3, -6, -7, and -9 mRNA and protein levels. Caspase mRNA expression was increased already after 2 days of ATRA treatment and reached a maximum induction after 6 days (Fig. 4A). Western blot analysis confirmed that caspase proteins also were strongly up-regulated after 4 and 6 days of the initiation of ATRA treatment (Fig. 4B).

Chemical Inhibition of p53 or Caspases Prevents UV-Induced Apoptosis of ATRA-Treated KCs. To test the involvement of p53 and caspases in the UVB-induced cell death, the pharmacologic inhibitors α-pifithrin (37) and z-VAD-fmk, respectively, were added to the KC cultures immediately after UVB treatment. Although not completely preserving normal cell morphology, z-VAD-fmk abolished detachment of KCs (Fig. 5A), blocked activation of caspase-3 (Fig. 5B), and prevented DNA fragmentation (Fig. 5C). α-Pifithrin also reduced cell detachment (Fig. 5A), caspase-3 activation, and chromatin condensation (Fig. 5B) and strongly but not completely reduced DNA fragmentation (Fig. 5C). These data confirm that p53 and caspases are involved in UVB-mediated apoptosis of confluent ATRA-treated KCs.

ATRA-Treated KCs Are Highly Sensitive to Doxorubicin-Induced Apoptosis. The chemotherapeutic agent doxorubicin induces DNA damage (38) and leads to programmed cell death by a p53-dependent mechanism (39), and we found that doxorubicin treatment of confluent KCs caused apoptosis only if they were treated with ATRA as confirmed by morphologic criteria (data not shown) and DNA fragmentation (Fig. 5D). The apoptotic effects of doxorubicin also could be blocked by the addition of z-VAD-fmk and α-pifithrin (Fig. 5D). These results show that ATRA treatment also sensitizes KCs to non–UVB-dependent, p53/caspase-mediated apoptosis.

ATRA Treatment Leads to Up-Regulation of p53 and Proapoptotic Caspases in KCs of SEs. To test whether similar observations could be found in the more in vivo-like SEs, we analyzed the effect of retinoids in SEs and found in analogy to monolayer culture that ATRA treatment led to an increased expression of p53 but not of p63 (Fig. 6A) and to enhanced expression of caspases (Fig. 6B). Immunofluorescence staining revealed that p53 was strongly expressed in all of the layers after ATRA treatment (Fig. 6C, d), whereas in controls it was only detectable in basal cells (Fig. 6C, c). In contrast,
ATRA increases UVB-induced apoptosis in KC

Fig. 6. p53 and caspases are up-regulated by ATRA and contribute to UVB-induced apoptosis in SEs. SEs were analyzed for the expression p53 and p63 using Western blot analysis (A) or for expression of caspase-3, -6, -7, and -9 using real-time PCR (B) (*, \( P < 0.05 \)). Morphology of SEs cultured in the absence (C, a, c, and e) or presence (C, b, d, and f) of ATRA was analyzed by H&E staining (C, a and b). Expression of p53 (C, c and d) and p63 (C, e and f) was analyzed by immunofluorescence staining; bar = 50 \( \mu \)m. D, SEs were cultured in the absence or presence of 1 \( \mu \)mol/L ATRA for 6 consecutive days. On day 6, UVB irradiation (100 mJ/cm\(^2\)) was carried out in the presence or absence of z-VAD-fmk (40 \( \mu \)mol/L) or \( \alpha \)-pifithrin (90 \( \mu \)mol/L). Twenty-four hours after UVB irradiation, SEs were stained for active caspase-3 (red) or nuclei (blue); bar = 50 \( \mu \)m.

p63 could be detected only in basal KCs in both groups (Fig. 6C, e and f). Z-VAD-fmk and, to a lesser extent, \( \alpha \)-pifithrin also blocked UVB-induced apoptosis in ATRA-treated SEs (Fig. 6D). These data show that ATRA sensitization of KCs to apoptosis also is accompanied by up-regulation of p53 and proapoptotic caspases in SEs.

DISCUSSION

Retinoids have been a mainstay of therapy for psoriasis for several decades (2). Retinoids more recently have been successfully used to prevent NMSC in high-risk individuals, such as patients with xeroderma pigmentosum (3) and transplant recipients receiving immune suppressive therapy (4). Whereas the beneficial effect of retinoids on psoriasis is most likely because of their potential to modulate KC differentiation (6), their mode of action in tumor prevention is not fully understood. It was suggested that regulation of activator protein signaling might be involved (5). However, the fact that retinoids are unable to control growth of already existing carcinomas while effectively preventing the appearance of new ones (40), together with the observation that activator protein acts similarly in primary KCs and in squamous cell carcinoma lines (41), argues against an exclusive role of this transcription factor. Here we show for the first time that ATRA treatment sensitizes KCs to apoptosis induced by UVB and by the DNA-damaging agent doxorubicin. This proapoptotic effect is accompanied by induction of the tumor suppressor protein p53 and the induction of proapoptotic caspases.

The fact that despite the strong up-regulation of p53 and proapoptotic caspases no spontaneous apoptosis of retinoid-treated KCs was observed in monolayer cultures is in agreement with the previous observations that even strong overexpression of caspases or p53 does not necessarily cause apoptosis but rather sensitizes cells to apoptotic cell death (19, 20). This notion is supported at the molecular level by the observation that in the absence of apoptotic stimuli, the p53 target gene \( MDM2 \) is up-regulated, whereas protein accumulation of p53 and strong up-regulation of the proapoptotic target gene \( Noxa \) were present only after additional exposure to UVB. The parallel increase of p53 and several caspases, together with the previous observation that p53 can increase the transcription of caspases (27, 42), suggests a connection between these two events. However, the temporal pattern (i.e., up-regulation of caspases before up-regulation of p53) shows that in addition to p53, other pathways may be responsible for the increased expression of caspases.

The involvement of p53 and caspases in apoptosis of retinoid-treated KCs could be supported by the reduction or abrogation of apoptosis using the p53 inhibitor \( \alpha \)-pifithrin or the caspase-inhibitor z-VAD-fmk. Unfortunately, it recently became evident that \( \alpha \)-pifithrin is not an exclusive inhibitor of p53 but also is able to inhibit heat shock and glucocorticoid signaling pathways (43). Therefore, we cannot formally exclude that a p53-independent antiapoptotic effect of \( \alpha \)-pifithrin may play an additional role in our experimental system. Because of the difficulty of efficiently transfecting highly confluent primary KCs and the loss of inhibitory activity 4 days after transfection, we could not achieve transfer of specific RNA interference at acceptable efficiency in our setting, thus precluding the use of this specific gene knockdown approach.

The effect of retinoid treatment on the sensitivity of KCs to UVB-induced apoptosis and p53 expression has been addressed previously by Li et al. (44), who reported that no influence on either parameter was found. We found that the proapoptotic effect of retinoids is evident only in confluent but not in preconfluent KCs. Because Li et al. (44) do not detail their protocol for KC cultures, we can only assume that the dissenting data may be caused by different culture conditions.

With regard to a possible causal relationship between p53 increase and suppression of KC differentiation, it was shown in the past that retinoids influence p53 expression in several cell types (45, 46) and that this was associated with a modulation of differentiation. Because ATRA inhibition of terminal KC differentiation and the maintenance/increase of p53 expression occurred simultaneously, we cannot rule out at the moment that a causal link exists between these two events. Arguments for a lack of involvement and evidence for the participation of p53 in terminal KC differentiation have been reported. Arguments against such a role come from studies that show that neither mice with deletions of the p53 gene (18) nor mice overexpressing a mutant activated form of p53 (47) show epidermal abnormalities. Conversely, the finding that overexpression of p53 in human KCs in vitro delays expression of differentiation-associated proteins argues for a direct link (48). A way to address this question in our experimental setting would be the silencing of p53 in KCs (e.g., by using

1 Unpublished observation.
retinoids may play a role in the prevention of UV-associated NMSC in patients with immunosuppressive therapies and DNA-repair defects.

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