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Inflammation Due to Voriconazole-induced Photosensitivity Enhanced Skin Phototumorigenesis in *Xpa*-knockout Mice

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Keywords; Voriconazole, Photosensitivity, Skin tumorigenesis, *Xpa*-knockout mice

Abbreviations: Xpa, Xeroderma pigmentosum complementation group A; ROS, reactive oxygen species; 8-oxoGua, 7,8-dihydro-8-oxoGuauanine; CPD, cyclobutane pyrimidine dimer; HCTZ, hydrochlorothiazide; VN-oxide, voriconazole N-oxide

ABSTRACT

Voriconazole is an anti-fungal agent and used as a prophylactic measure, especially in immunocompromised patients. However, there have been several reports of its adverse reactions, namely photosensitivity with intense inflammatory rashes and subsequent skin cancer development. To assess the effects of photosensitizing drugs voriconazole and hydrochlorothiazide (HCTZ) on the enhancement of UV-induced inflammatory responses and UV-induced tumorigenesis, we utilized *Xpa*-knockout mice, which is DNA repair-deficient and more susceptible to UV-induced inflammation and tumor development than wild-type mice. Administration of voriconazole prior to broad-band UVB exposure significantly upregulated multiple inflammatory cytokines compared with the vehicle- or HCTZ-administered groups. Voriconazole administration along with chronic UVB exposure produced significantly higher number of skin tumors than HCTZ or vehicle in *Xpa*-knockout mice. Furthermore, the investigation of UVB-induced DNA damage using embryonic fibroblasts of *Xpa*-knockout mice revealed a significantly higher 8-oxo-7,8-dihydroguanine level in cells treated with voriconazole N-oxide, a voriconazole-metabolite during UV exposure. The data suggest that voriconazole plus UVB-induced inflammatory response may be related to voriconazole-induced skin phototumorigenesis.

INTRODUCTION

Voriconazole is a second-generation triazole antifungal agent that is effective against a wide range of fungal infections, such as fluconazole-resistant candidiasis, aspergillosis, coccidioidomycosis and prescribed as a prophylactic agent for immunosuppressed individuals, such as organ transplant recipients or chemotherapy patients (1). However, a number of side effects have been reported, including photosensitivity reactions such as facial erythema and cheilitis, photoaging (indicated by solar lentigines in sun-exposed areas of the skin) (2) (3). Subsequently, non-melanoma skin cancer and melanoma may develop in the sun-exposed area of the skin after a long-term voriconazole administration (4), featuring multiple squamous cell carcinomas and their precursor lesions, actinic keratosis, distributed over the face and neck (5, 6). We previously showed that UVB-induced inflammatory response was highly associated with DNA damage caused by UVB, such as 8-oxo-7,8-dihydroguanine (8-oxoGua) and cyclobutane pyrimidine dimers (CPDs) by using *Ogg1* knock-out mice or *Xpa*-knockout mice, which are deficient in repairing DNA damage, 8-oxoGua or CPDs, respectively Namely, *Ogg1*-knockout mice and *Xpa*-knockout mice produced more intense UV-induced inflammatory response and expressed much higher inflammatory cytokines such as CXCL1 (7) (8). (9). The study has also shown that *Ogg1*-knockout mice and *Xpa*-knockout mice are more susceptible to UV-induced tumorigenesis. Furthermore, CXCL1, a neutrophil attractant

chemokine, is significantly upregulated in the blood serum of *Xpa*-knockout mice after UVB exposure and once the neutralizing antibody against CXCL1 was administered at every exposure of UVB, the number of developed skin tumors in *Xpa*-knockout mice actually decreased (8). Long-term use of the diuretic agent hydrochlorothiazide (HCTZ), another photosensitizing drug, was associated with an increased risk of non-melanoma skin cancer (10) (11) and we previously showed that HCTZ enhanced the formation of CPD after UVA irradiation (12). Taken together, the abnormal photosensitized skin inflammation caused by voriconazole or HCTZ may play a role in the development of non-melanoma skin cancers. To determine the mechanisms of enhancement of photo-induced skin cancer by voriconazole, in the present study, we investigated the effects of voriconazole and HCTZ on the enhancement of inflammatory reaction by profiling cytokines and skin tumor development in *Xpa*-knockout mice and wild-type mice.

MATERIALS AND METHODS

Mice and Reagents. *Xpa*-knockout mice (*Xpa*-deficient mice) with CBA (Nakane *et al.*, 1995), C57BL/6, and CD-1 chimeric backgrounds were backcrossed with hairless albino mice from the Balb/cA Kud-hr inbred strain. The resulting hairless *Xpa* (+/+) and *Xpa* (-/-) mice aged 9-12 weeks

were used. The mice were housed under specific pathogen-free conditions, and all experiments using these animals were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine. Voriconazole N-oxide and HCTZ were obtained from Santa Cruz Biotechnology (Dallas, TX) and Wako (Osaka, Japan), respectively. Owing to its low solubility in saline, we purchased voriconazole from a pharmaceutical company as a distribution source.

UVB irradiation and observation of tumor incidence. A bank of six TL 20W/12RS fluorescent lamps (Philips, Eindhoven, Holland), which emit a continuous spectrum from 275-390 nm, with a peak emission at 313 nm, was used to irradiate the mice (13) (14). To observe the development of skin tumor, the mice were placed 40 cm below the light source and irradiated with UVB at 0.25 kJ/m^2 and 1.50 kJ/m^2 for *Xpa*-knockout mice and wild-type mice, respectively, twice a week for 10 weeks. 1.50 kJ/m^2 was the approximate minimal erythema dose required to cause faint, but discernible erythema in Kud hairless background wild-type mice (15). After 10 weeks of chronic UVB exposure, we observed tumor development for another 10 weeks. To administer the drugs to the mice, intraperitoneal injections of voriconazole (0.5 mg/mouse) and HCTZ (0.1 mg/mouse) were performed twice per week, 2 h before UVB irradiation, for 10 weeks and subsequently discontinued during the 10 week observation period for the development of skin tumors. Only the developed

tumors with diameters larger than 2 mm were counted. The tumors in mice that died during the experiment were not included.

Cytokine detection assay. For the detection of cytokine expression, 48 h following irradiation with 600 kJ/m^2 of 365 nm-peaked narrow-band UVA or 1.0 kJ/m^2 of broad-band UVB and intraperitoneal injection of either voriconazole, HCTZ, or vehicle, serums were isolated from blood samples and subjected to ELISA assay according to the manufacturer's instructions (R&D, Minneapolis, MN).

DNA damage detection. Mouse embryonic fibroblasts (MEFs) were prepared from E13.5 embryos of *Xpa*-knockout and wild-type mice (8). The MEFs of each *Xpa* genotype were exposed to 0.20 kJ/m^2 broad-band UVB following the addition of 10 μg/mL voriconazole, 10 μg/mL voriconazole N-oxide (VN-oxide), or 10 ng/mL HCTZ in PBS and incubation until the time point of corresponding DNA isolation. DNAs were then subjected to 8-oxoGua-ELISA (Trevigen, Gaithersburg, MD) and CPD-ELISA (Cosmo Bio, Tokyo, Japan) to quantify the 8-oxoGua (ng/mL) and CPD, respectively.

Statistical analysis. The differences in cytokine expressions, the number of developed tumors, and DNA damage between the two groups were assessed for significance using the Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

To investigate the effects of photosensitive inflammation due to voriconazole or HCTZ on and to determine the cytokines involved in those responses, we utilized a mouse cytokine panel capable of detecting the expression of forty cytokines simultaneously. *Xpa*-knockout and wild-type mice were exposed to 600 kJ/m^2 of 365-nm-peaked narrow-band UVA (16) and 1.0 kJ/m^2 broad-band UVB (14), with the administration of voriconazole, HCTZ, or vehicle prior to UV exposure. Blood samples were taken 48 h after UV irradiations and subsequently subjected to ELISA. Despite no significant changes in UVA-induced cytokine expression with any drug treatment in both *Xpa*-knockout and wild-type mice, the *Xpa*-knockout mice irradiated with UVB plus photosensitizing drugs showed significant upregulation of cytokines compared with wild-type and UVB-only controls (Figure 1a). A unique pattern emerged where some cytokines were exclusively and simultaneously up-regulated in voriconazole-administered and in HCTZ-administered mice with relatively lower expression of HCTZ-administered mice than that of the voriconazole-administered mice (Figure 1a). These results indicate that the responsible wavelength for the enhancement of UV-induced cytokine expression by these drugs fall into the 275-390 nm, which is consistent with

the absorption spectrum of HCTZ or voriconazole and its major hepatic metabolite, VN-oxide (16) (17). Furthermore, we studied whether there was a possibility that voriconazole itself, in the absence of UV, was involved in some effects on the up-regulation of cytokines. However, no obvious effects in the voriconazole or HCTZ only groups, in the absence of UV irradiation, were observed for either *Xpa* genotype (Supplementary Figure). Thus, the up-regulation of multiple cytokines by voriconazole or HCZ was enhanced only when accompanied by UV exposure. Based on the results that showed multiple cytokine up-regulation in *Xpa*-knockout mice by broad- band UVB lamp, not by narrow-band UVA lamp, thus, we studied the voriconazole-induced enhancement of skin tumor development in *Xpa*-knockout mice after chronic UVB exposure. The UVB plus voriconazole group showed significantly higher number of skin tumors at the end of the experiments as well as faster progression of tumor development than either UVB plus HCTZ or UVB-only group (Figure 1b).

To date, several mechanisms of voriconazole-induced photosensitivity leading to the development of skin cancers have been proposed. Ona *et al.* reported that VN-oxide, the major hepatic metabolite of voriconazole, is responsible for forming UVB-photoproduct, which exerts photosensitivity (cell killing) to UVA, and generate reactive oxygen species (ROS) and subsequently produce 8-oxoGua (18). Ikeya *et al.* showed that voriconazole metabolite plus UVA generated ROS and resultant DNA

damage, but did not induce substantial apoptosis in human keratinocytes, while voriconazole per se stimulates aryl hydrocarbon receptor and up-regulated cyclooxygenase 2, which is thought to have a major role in UV-induced inflammatory elements. *Xpa*-knockout mice, which is impaired in repairing ROS-related molecules (19), were probably strongly stimulated by the aryl hydrocarbon receptor-dependent, namely classical (genomic) pathway, and associated with the development of skin cancers. (20,21). To examine the effects of voriconazole, VN-oxide, and HCTZ on the amount of DNA damage after only UVB-wavelength exposure, we measured and compared CPD and 8-oxoGua formation among the broad-band UVB only, broad-band UVB plus either voriconazole, VN-oxide, or HCTZ, and control groups (drug only) using MEFs of *Xpa*-knockout and wild-type mice. Despite no significant difference in CPD formation among the analyzed groups at either time point after UVB irradiation, the production of 8-oxoGua in VN-oxide plus UVB treatment group was significantly higher than that of UVB alone 24 h after UVB exposure in *Xpa*-knockout mice MEFs (Figure 2). These data indicate that ROS-induced 8-oxoGua formation and subsequent cytokine upregulation, possibly caused by UVB absorption by VN-oxide were involved in the increased development of skin tumors in *Xpa*-knockout mice. These mice, which have revealed substantial ROS production by UVB (22), manifested more visible voriconazole-induced

photosensitivity and phototumorigenesis, whereas wild-type mice did not show any significant difference when compared with groups administered voriconazole, HCTZ, and vehicle, although we had expected photocarcinogenic effects of voriconazole in wild-type mice similar those seen in humans (data not shown). Because *Xpa*-KO mice was reported to present much more UV-induced immune suppression than wild type (23), it is possible that UV plus voriconazole in *Xpa*-knockout mice contributes to immunosuppression as it does in humans; voriconazole-prescribed patients tend to also receive a combination of several immunosuppressive agents. However, in our experimental conditions, IL-10 expression, even in *Xpa*-knockout mice, was below the level of detection (Figure 1a) (24). Therefore, the immunomodulatory effects of UVB and voriconazole in *Xpa*-knockout mice may not be so large. On the other hand, the definite association between non-melanoma skin cancer development in immunocompromised individuals and the usage of voriconazole was not conclusive after statistical adjustment for clinical factors (25). Furthermore, it should be clarified why a part of human population taking voriconazole develop skin cancer, although high proportion of voriconazole treated patients appear to show photosensitivity (26), and any tumor-susceptible effects of voriconazole in wild-type mice did not occur in this study. The polymorphism of *CYP2C19* gene, which codes a hepatic voriconazole-metabolizing enzyme in

humans, is thought to be associated with VN-oxide-forming fractions that could lead to a difference in skin reaction to UV (27). Although the human *CYP2C19* gene is not exactly analogous to the mouse *Cyp2c* gene and a specific study of *Cyp2c* polymorphism has not been performed so far (28), the present study suggests that the difference among mouse polymorphism is probably lesser than that of humans.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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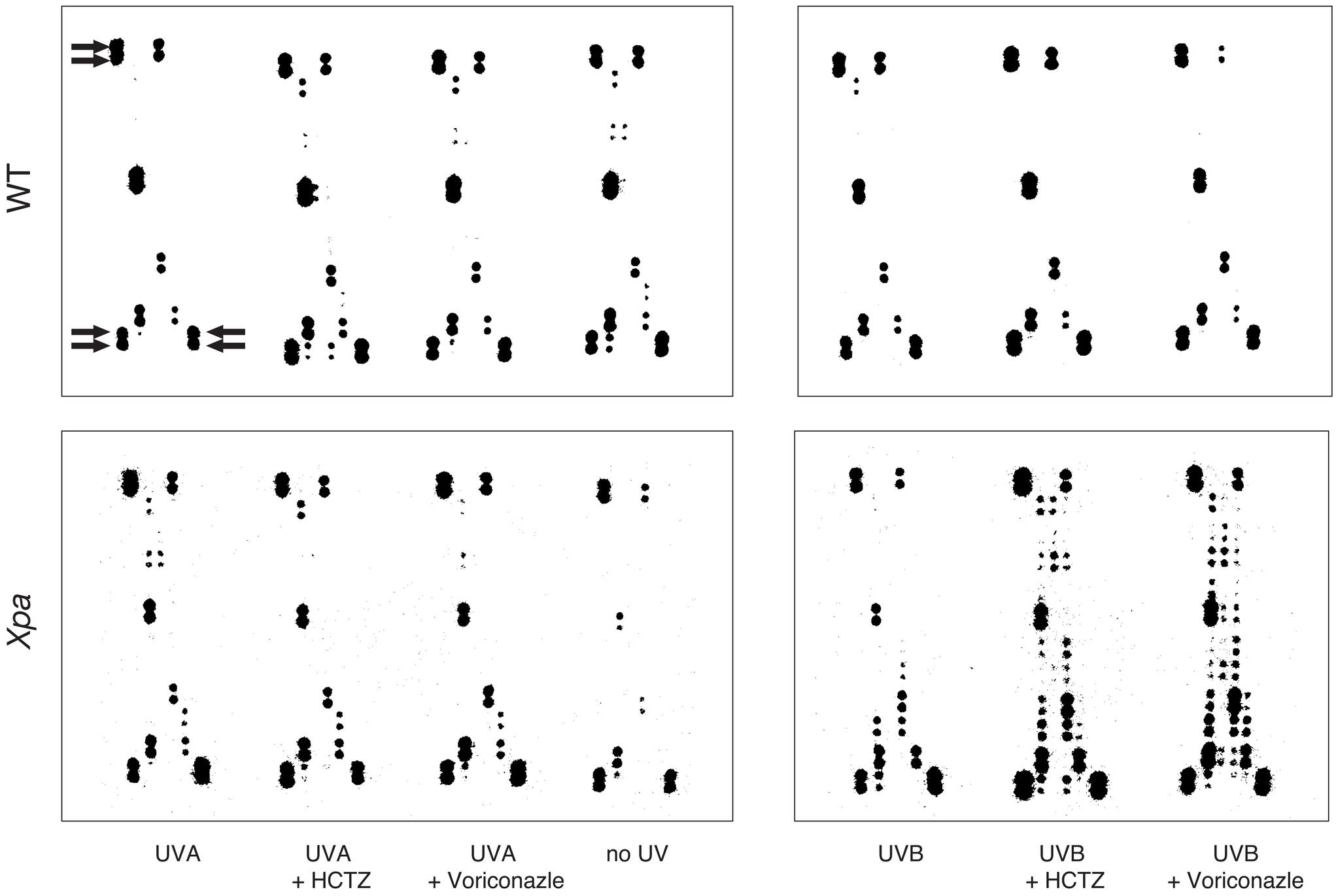
FIGURE LEGENDS

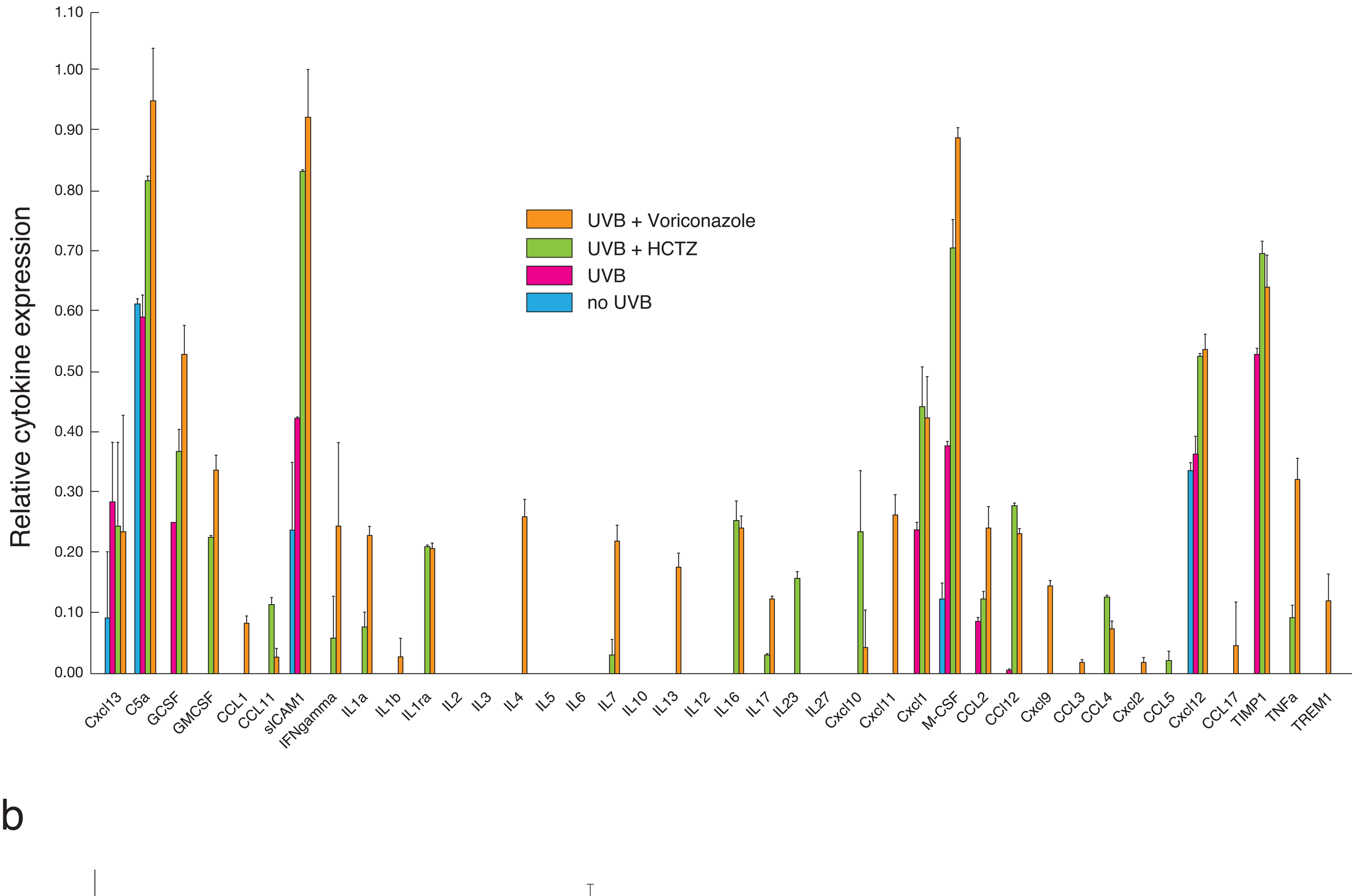
Fig. 1. (a) Upper panel: mouse cytokine array for *Xpa*-knockout and wild-type mice. Each spot representing cytokine expression was duplicated. The six arrows in the membrane indicate positions of the reference spots. Each squared panel represents simultaneous exposure to X-ray of different membranes. Lower panel: relative expression of forty cytokines in the membranes of *Xpa*-knockout mice after UVB exposure with voriconazole, HCTZ, and vehicle and in no-UV controls. The average intensities of duplicated membrane signals detected by X-ray (upper panel) were measured and normalized to reference spots. Semi-quantification of signals was performed using ImageJ software (NIH, Bethesda, MD). The expression is represented as mean ± SD. (b) The enhanced effects of UVB-induced tumorigenesis by voriconazole on *Xpa*-knockout mice. The left panel shows the average number of skin tumors/mouse (mean±SD), and the right panels show skin tumor incidence by treatment with voriconazole (n = 9), HCTZ (n = 9), and vehicle (n = 8) via intraperitoneal injection. *Xpa*-knockout mice were irradiated twice a week with 0.25 kJ/m^2 UVB for 10 weeks along with either treatment followed by observation of skin tumor development with no UVB exposure for another 10 weeks. The peritoneal injections of voriconazole (0.5 mg/mouse) and HCTZ (0.1 mg/mouse) were administered twice a week 2 h before UVB irradiation for 10 weeks. wk, week.

Fig. 2.

The formation of 8-oxo-7,8-dihydroguanine (8-oxoGua, upper graph) and cyclobutane pyrimidine dimers (CPDs, lower graph) in mouse embryonic fibroblasts (MEFs) derived from *Xpa*-knockout and wild-type mice subjected to different treatments. Each sample was duplicated, and the expression was represented as the mean ± SD. The asterisk indicates the difference in 8-oxoGua expression 24 h after UVB exposure between UVB only and plus VN-oxide treated *Xpa*-knockout mice-derived MEFs. *$P < 0.05$

a


WT
Xpa
UVA
UVA + HCTZ
UVA + Voriconazle
no UV
UVB
UVB + HCTZ
UVB + Voriconazle


Relative cytokine expression
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
UVB + Voriconazole
UVB + HCTZ
UVB
no UVB
Cxcl13
C5a
GCSF
GMCSF
CCL1
CCL11
sICAM1
IFNgamma
IL1a
IL1b
IL1ra
IL2
IL3
IL4
IL5
IL6
IL7
IL10
IL13
IL12
IL16
IL17
IL23
IL27
Cxcl10
Cxcl11
Cxcl1
M-CSF
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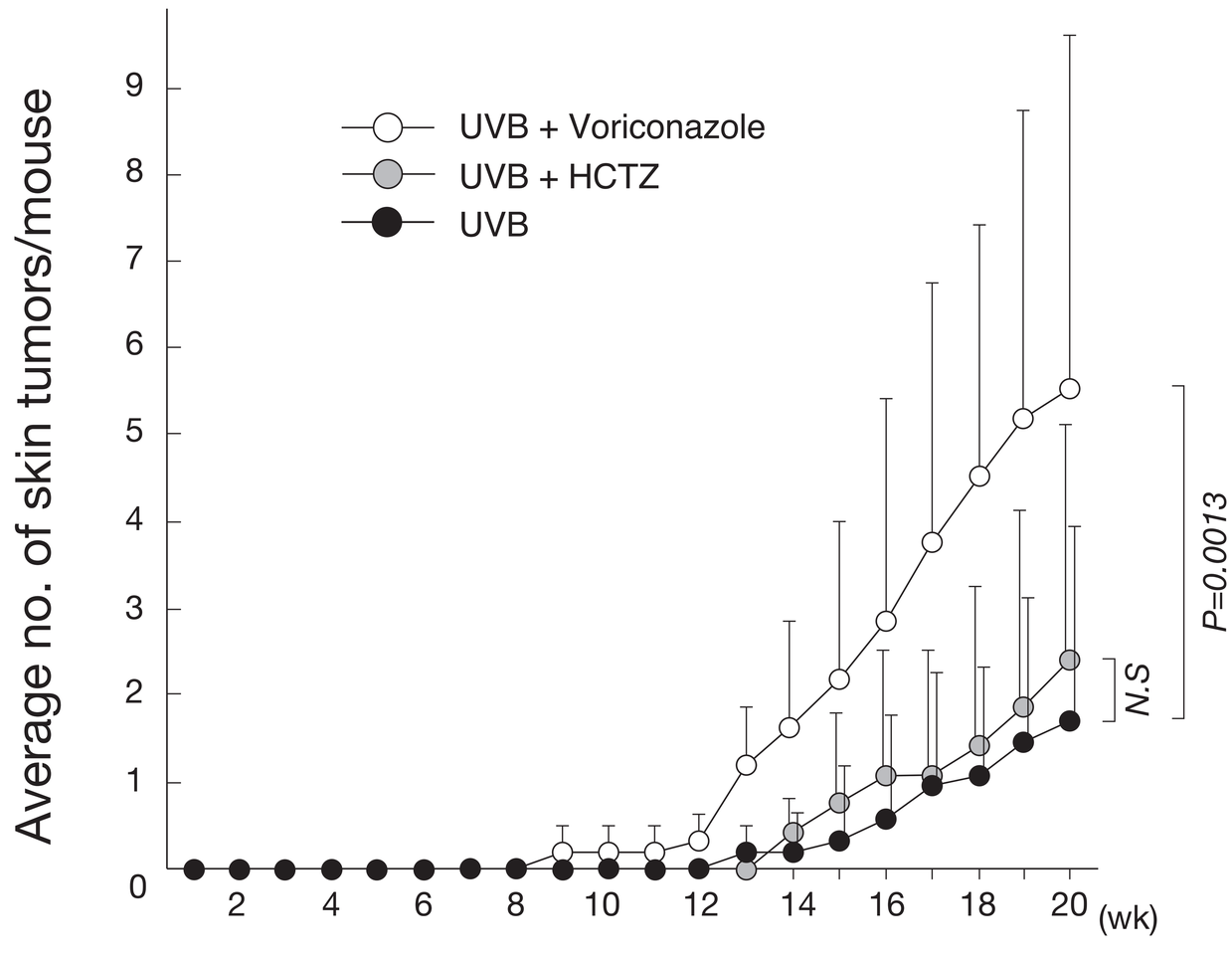
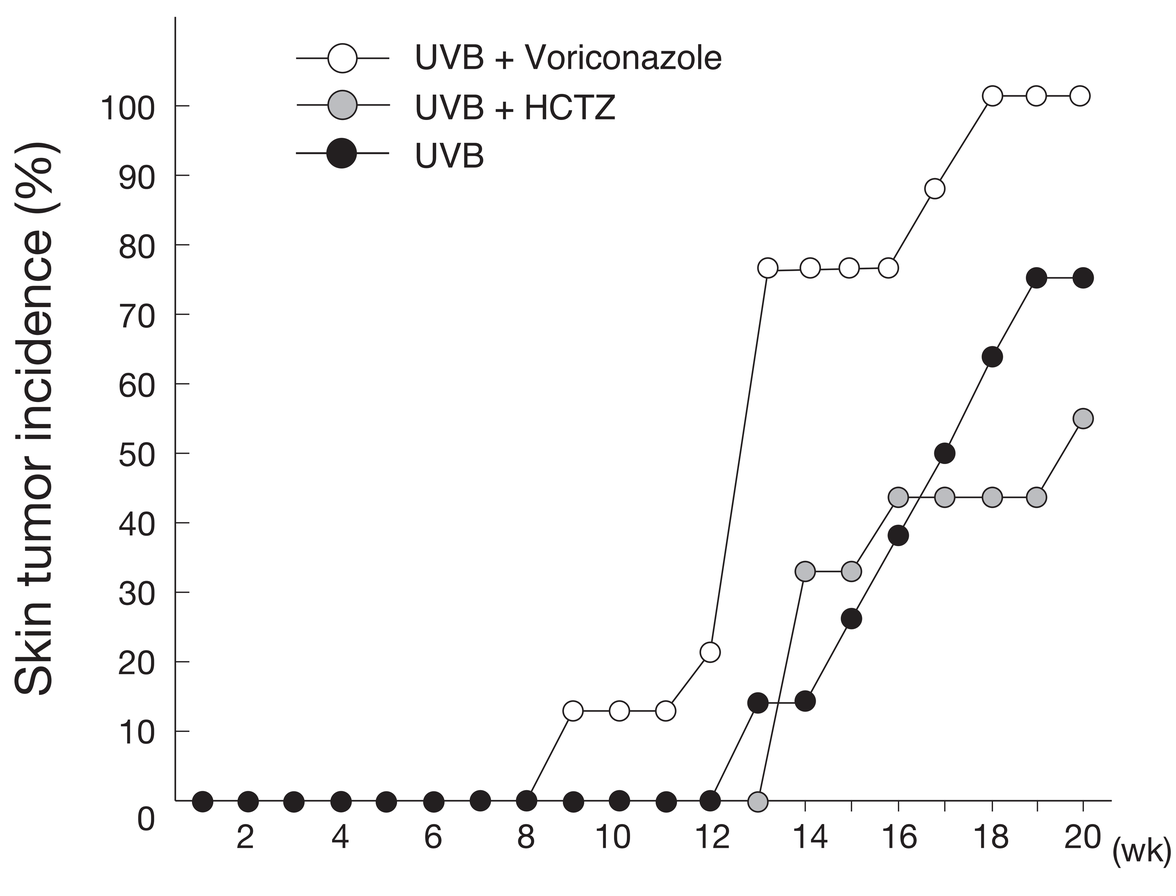
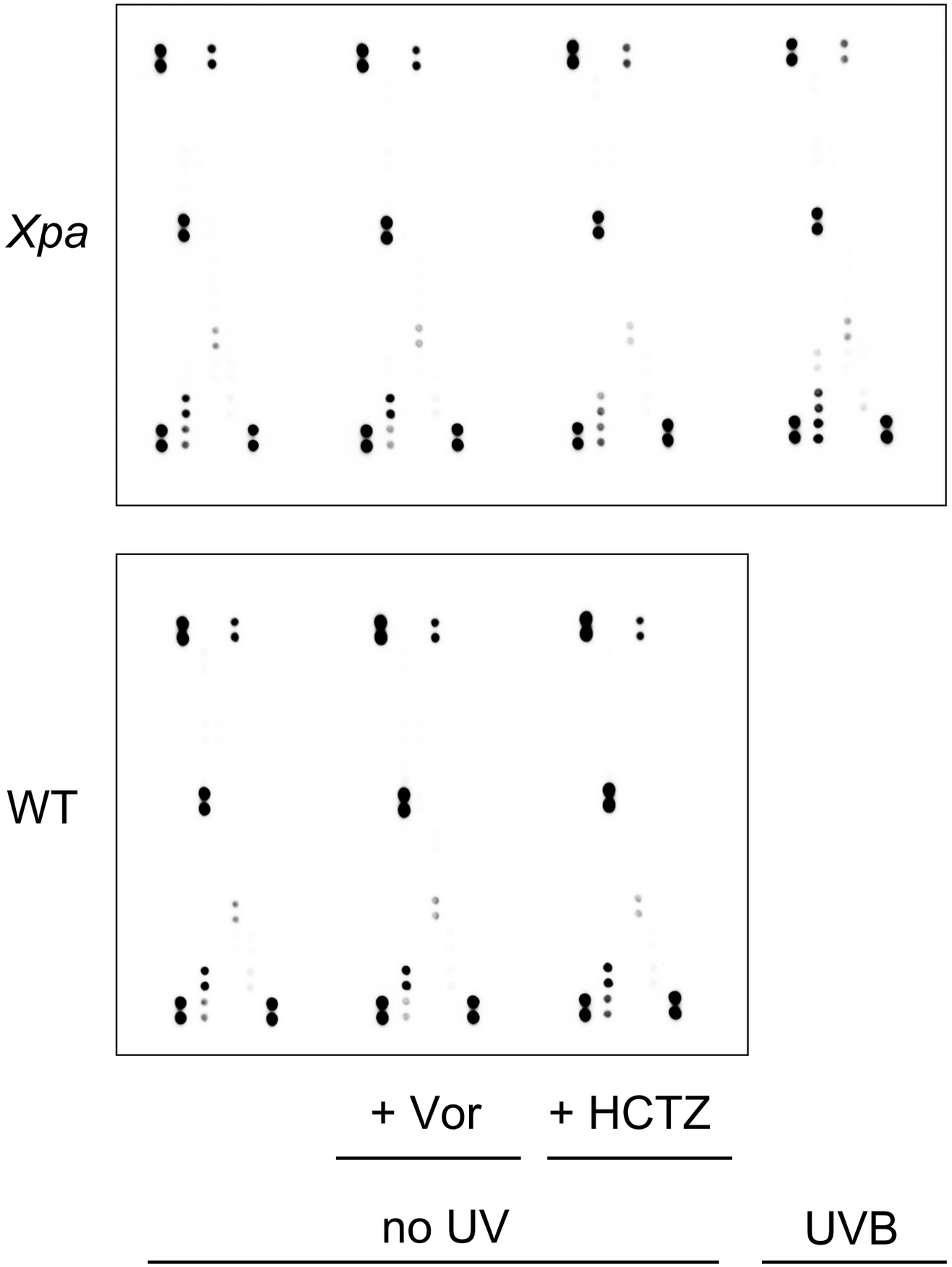
TNFa
TREM1

b


Average no. of skin tumors/mouse
9
8
7
6
5
4
3
2
1
0
UVB + Voriconazole
UVB + HCTZ
UVB
2
4
6
8
10
12
14
16
18
20
(wk)
P=0.0013
N.S


Skin tumor incidence (%)
100
90
80
70
60
50
40
30
20
10
0
UVB + Voriconazole
UVB + HCTZ
UVB
2
4
6
8
10
12
14
16
18
20
(wk)

UVB only
HCTZ
Voriconazole
VN-oxide
WT
Xpa
*
The yeilds of 8-oxoGua (ng/mL)
0.935
0.930
0.925
0.920
0.915
0.910
0.905
0.005
0.000
CPDs Absorbance
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
no UVB
1 min
3 hr
24 hr



A mouse cytokine array of *Xpa*-knockout and wild-type mice treated with drugs in the absence of UV. Forty-eight hours after the peritoneal injections of voriconazole (0.5 mg/mouse), HCTZ (0.1 mg/mouse) or vehicle to both wild-type and *Xpa*-knockout mice, blood samples were taken and subjected to a cytokine array using the same condition in Figure 1. As a reference, the blotting image of 48hr after UVB 100 mJ/cm^2 for *Xpa*-knockout mice is shown. Each cytokine expression spot appears in duplicated.