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A.Y.: performed the experiments. A.Y., K.F. and T.K.: substantially contributed to the conception and design of the work, interpretation of data, and to drafting and revising

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Abstract up to 250 words = 199 words

Detailed mechanisms on the effect of oxidative stress (OS), an etiological factor involved in photocarcinogenesis, remain to be fully elucidated. We used immunohistochemial methods to study OS in the DNA, protein, and lipid of patients with actinic keratosis (AK), Bowen's disease (BD), and squamous cell carcinoma (SCC). Between January 2009 and December 2014, we treated 230 patients; 79 had AK, 61 had (BD), and 90 had cutaneous SCC; 28 healthy subjects served as the normal control. OS on DNA, protein, and lipid was assessed by the expression of 8-hydroxydeoxyguanosine (8-OHdG), dityrosine (DT), and malondialdehyde (MDA), respectively. 8-OHdG was significantly overexpressed in AK and BD lesions compared with surrounding non-lesional tissue, SCC lesions, and the healthy controls. DT was more highly expressed in AK, BD, and SCC than in the controls. There was no significant difference among AK, BD, and SCC. The expression of MDA was higher in AK, BD, and SCC lesions than the controls; SCC showed the highest expression. Our observations suggest that DNA oxidation plays an important role in the early stage of carcinogenesis, that protein oxidation is involved in all stages of carcinogenesis, and that lipid oxidation is strongly implicated in the late stages of carcinogenesis.

Introduction

Squamous cell carcinoma (SCC) is the most common skin cancer in humans. It can develop anywhere on the skin and mucous membranes covered by squamous epithelium but has a predilection for sun-damaged skin.[1)] Pre-cancerous lesions include burn scars, stasis ulcers, and radiation dermatitis.[1)] The incidence of metastasis is 2 - 3% in all patients with SCC of the skin; the mortality rate is approximately 75% in patients with metastasis.[2) 3)] Metastasis from SCC arising in sun-damaged skin is relatively rare; its incidence is approximately 0.5%[4] and tends to be curable by surgery. Oxidative stress (OS) has been studied as a contributor to aging and carcinogenesis and was reported to be related to esophageal-,[5)] non-small-cell lung-,[6)], and skin cancer.[7)] The endogenous production of free radicals by the cellular metabolism and from exogenous sources such as ultraviolet radiation (UVR) and pollution can damage the skin on the cellular and tissue level.[8)] Arsenic is a representative pollutant underlying the development of Bowen's disease (BD) and SCC.[9)] By producing OS, UVR plays a major role in skin aging and the development of skin cancers.[8) 10)] However, there is little *in vivo* information on the role of OS in skin carcinogenesis.

We used immunohistochemical methods to examine the expression of OS-related molecules in the course of SCC carcinogenesis. DNA damage and protein- and lipid oxidation were evaluated by the expression of 8-hydroxydeoxyguanosine (8-OHdG), dityrosine (DT), and malondialdehyde (MDA), in specimens of actinic keratosis (AK), BD, and SCC.

Materials and Methods

Patients and preparation of specimens

This study was approved by the Human Research Ethics Committee of our institution; prior informed consent was obtained from all participants.

We obtained tissue samples by biopsy or surgery from 28 healthy controls and from 230 consecutive AK 79, BD 61, or SCC 90 patients treated between January 2009 and December 2014. The study subjects were 131 men and 127 women, the median age was 78.6 years (range 42 - 104 years). Patient data are shown in Table 1.

Samples were obtained from the most invasive area of each lesion. They were fixed in 10% formalin solution, embedded in paraffin, and cut into 5-μm-thick slices. One section was counterstained with hematoxylin and eosin (H&E) for histological diagnosis and stained immunohistochemically.

Immunohistochemical staining

We immunostained adjacent serial sections for 8-OHdG, DT, and MDA to evaluate the OS response of DNA, protein, and lipid. Sections (thickness 5μm) were deparaffinized in xylene and then rehydrated in a graded series of ethanol. To block endogenous peroxidase activity, the next steps were performed at room temperature (RT). For 8-OHdG assessment, the sections were treated for 5 min with 0.03% hydrogen peroxide in distilled water (DW). The expression of DT and MDA was assessed by 5- and 10-min treatment with 0.03% hydrogen peroxide in methanol, respectively. For 8-OHdG and DT, the sections were then exposed (8 min, 37ºC) to proteinase K (Dako, Copenhagen, Denmark). For MDA, specimens were incubated with 5% normal horse serum in phosphate-buffered saline (PBS) (Vector Laboratories,

Burlingame, CA, USA) (20 min, RT) to block any nonspecific binding of the immunoreagent. Subsequently, they were reacted for 1 hr at RT with anti-8-OHdG monoclonal antibody diluted to 1:50, anti-DT monoclonal antibody diluted to 1:100, or anti-MDA monoclonal antibody diluted to1:200 (all from Japan Institute for the Control of Aging, Shizuoka, Japan). For the assessment of 8-OHdG and DT, the binding of secondary antibodies was detected using the Dako EnVision/HRP system (Dako) (30 min, RT). For MDA, specimens were incubated (20 min, RT) with 1% biotinylated anti-mouse IgG serum in PBS (Vector); assessment was with the labeled avidin-biotinylated peroxidase complex technique (Vector) (30 min, RT). Color was developed with 3,3-diamino-benzidine (Vector).

Histological evaluation

We randomly selected three areas on each immunostained section and recorded the expression level of 8-OHdG, DT, MDA at least twice (magnification x 200). Immunoreactivity grading for 8-OHdG, DT, MDA was based on Remmele et al.[11] Staining intensity was rated as 0 (no)-, 1 (weak)-, 2 (moderate)-, and 3 (strong) staining. Stain-positive and negative cells were counted and the percentage of positive cells was scored as 0 (no)-, as 1 (1 - 50%), and as 2 (51 -100%).[5] The final immunoreactivity score (IRS) was calculated by multiplying the staining intensity score by the percentage of positive cells; theoretically, the results ranged from 0 to 6. To compare the intensity score with clinicopathological factors, a final score of 0 - 2, 3 - 4, and 5 - 6 was recorded as weak-, moderate-, and strong immunoreactivity, respectively. Histological and immunohistochemical evaluations were performed at least twice without information on the patient characteristics.

Statistical analysis

The Kruskal-Wallis nonparametric- and the Steel-Dwass test was performed for inter- and intra-group comparisons, respectively. Data are expressed as the mean ± standard deviation. Differences of $p < 0.05$ were considered to be statistically significant.

RESULTS

Expression of 8-OHdG, DT, and MDA

As shown in Figure 1A, 8-OHdG was overexpressed in AK and BD lesions. Representative stains for 8-OHdG in AK-, BD-, and SCC samples are presented in Figure 1B; staining was observed primarily in the nuclei of tumor cells. The expression level of DT was higher in AK, BD, and SCC than the control; there was no significant difference among the lesions (Fig. 2A). DT was mainly observed in the cytoplasm of tumor cells (Fig. 2B). MDA expression was higher in AK, BD, and SCC than the control. The IRS was higher in SCC than AK and BD (Fig. 3A); MDA was mainly observed in the cytoplasm and partially in the nuclei of tumor cells (Fig. 3B).

Comparison of IRS by clinical parameters

Comparison of the expression level of 8-OHdG, DT, and MDA in skin that was or was not exposed to the sun, the presence or absence of metastasis, and the presence or absence of old burn scars revealed no significant differences.

Discussion

OS is strongly associated with carcinogenesis and intracellular DNA, protein, and lipid are affected by OS. To assess cellular damage by OS, we recorded the expression of OS markers of DNA, protein, and lipid by immunohistochemical staining of AK-, BD-, and SCC samples.

Oxidative damage to DNA elicits mutagenesis and carcinogenesis.[12)] 8-OHdG, an oxidized form of deoxyguanosine nucleoside, is produced by oxidative damage to

DNA and can be detected by immunohistochemical analysis.[13] The expression of 8-OHdG was higher in human esophageal cancer tissue than in normal epithelium,[5] and predictive of the disease outcome.[14] The expression level of 8-OHdG was also prognostic in patients with non-small-cell lung- and urinary bladder cancer.[6,15]. In skin, 8-OHdG expression is induced by UVR in normal human epidermis[16] and increased in arsenic-induced BD.[17] We document that 8-OHdG is overexpressed in AK and BD tissue compared to adjacent non-lesional tissue, healthy controls, and SCC.

DT, formed by the oxidation of tyrosine in protein and free tyrosine, is a protein oxidation marker. It is increased in rabbit atherosclerotic lesions[18], aged human brain,[19], the skin epidermis of hairless mice with protein malnutrition,[20], and the kidney of rats with cisplatin-induced nephrotoxicity.[21] To the best of our knowledge, there is no direct evidence for the participation of DT in carcinogenesis. We found that the expression of DT was higher in AK, BD, and SCC than the control and that there was no significant difference among the three lesions. We suggest that protein oxidation is involved in all stages of SCC carcinogenesis.

MDA is a representative lipid peroxidation (LPO) marker. LPO leads to the formation of α,β-unsaturated aldehydes including MDA which was shown to be mutagenic and carcinogenic.[22] Excessive MDA combines with free amino acids and results in MDA-modified protein adducts, which can be used to assess OS-induced LPO.[23][24] MDA also reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine. This results in the production of pyrimidopurinone which is also mutagenic and carcinogenic.[24] When MDA was applied to the shaved backs of mice they developed carcinoma such as keratoacanthoma.[25] Sander et al.[26] reported that MDA was significantly increased in melanoma tissue. According to Williams et al.,[27] in

cultured human epidermal keratinocytes and healthy human skin, acute exposure to solar-simulated UV light led to the formation of free- and protein-bound MDA and dihydropyridine-lysine (DHP)-epitopes. Their tissue microarray analysis revealed that MDA and DHP epitopes are abundant in non-melanoma skin cancer compared to adjacent normal tissue. In our study, the expression of MDA was higher in AK, BD, and SCC than the control; it was higher in SCC than AK and BD. Our and earlier findings suggest that lipid peroxidation is associated with cancer progression and more highly implicated in its later stages.

Our results indicate that OS to DNA, protein, and lipid is involved in the development of cutaneous SCC and in different stages of skin carcinogenesis.

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Figure Legends

Figure 1

A. Box plots of the IRS for 8-OHdG in normal skin, AK, BD and SCC. 8-OHdG was overexpressed in AK and BD lesions.

   Red line: median. P-values were calculated with the Kruskal-Wallis test.

B. Representative stains for 8-OHdG in AK, BD, and SCC samples. Staining was strongest in the nuclei of tumor cells (original magnification x 200).

Figure 2

A. Box plots of the IRS for DT in normal skin, AK, BD, and SCC. The expression of DT was higher in AK, BD, and SCC than the control. There was no significant difference among AK, BD, and SCC.

   Red line: median. P-values were calculated with the Kruskal-Wallis test.

B. Representative stains for DT in AK, BD, and SCC samples. Staining was strongest in the cytoplasm of tumor cells (original magnification x 200).

Figure 3

A. Box plots of the IRS for MDA in normal skin, AK, BD and SCC. MDA expression was higher in AK, BD, and SCC than the control. It was highest in SCC samples.

   Red line: median. P-values were calculated with the Kruskal-Wallis test.

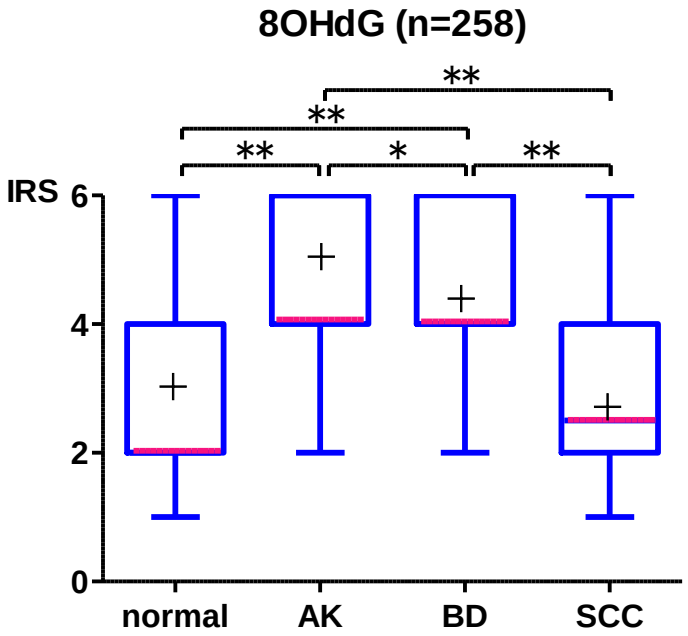
B. Representative stains for MDA in AK, BD, and SCC samples. Staining was observed mainly in the cytoplasm and partially in the nuclei of tumor cells (original magnification x 200).

Table 1. Clinical data on 258 studey subjects.

Clinical characteristics		Total (n=258)	Histological diagnosis			
			Normal control (n=28)	AK (n=79)	BD (n=61)	SCC (n=90)
Gender	Male	131	14	40	27	50
	Female	127	14	39	34	40
Age	Mean	78.6	70.7	80.5	76.6	80.8
	Range	42-104	42-94	49-104	55-99	46-100
Site	Head and neck	141	9	64	13	55
	Trunk	37	7	2	21	7
	Upper extremities	42	3	13	9	17
	Lower extremities	38	9	0	18	11

Abbreviations: n, number of cases; AK, actinic keratosis; BD, Bowen's disease; SCC, squamous cell carcinoma.

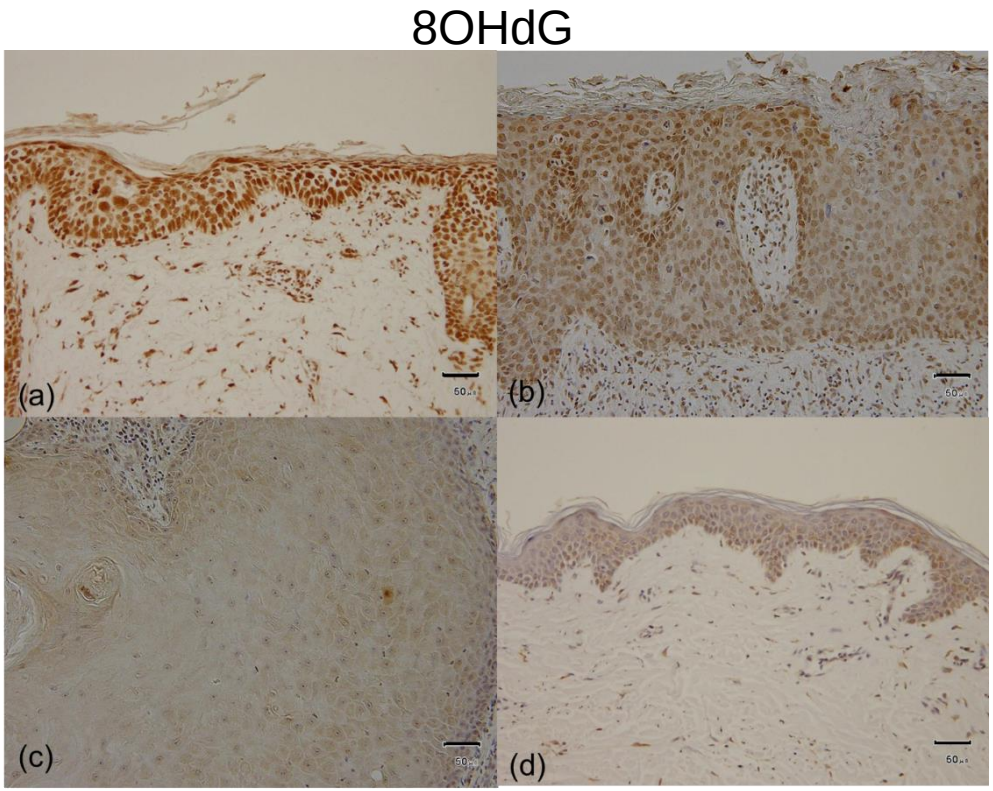
Fig.1A



(**P ≦ 0.01, *P ≦ 0.05)

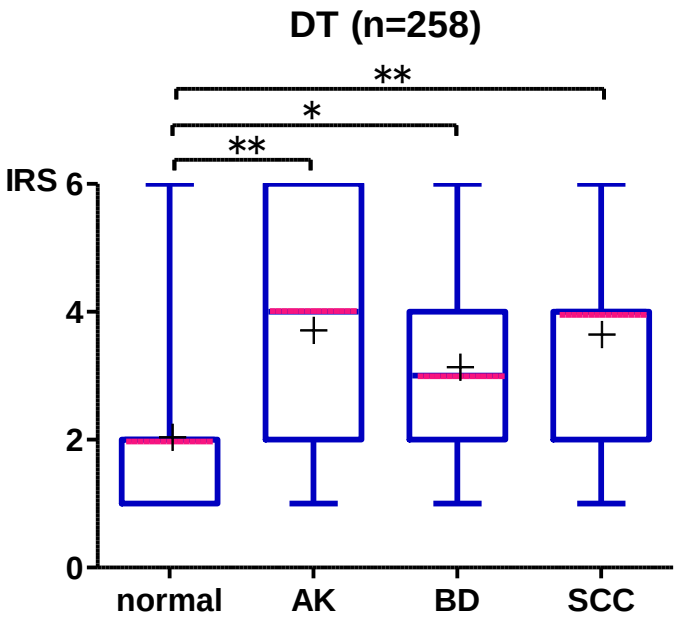
(＋： median)

Fig.1B



(a) AK 3×2	(b) BD 2×2
(c) SCC 1×2	(d) normal 2×1

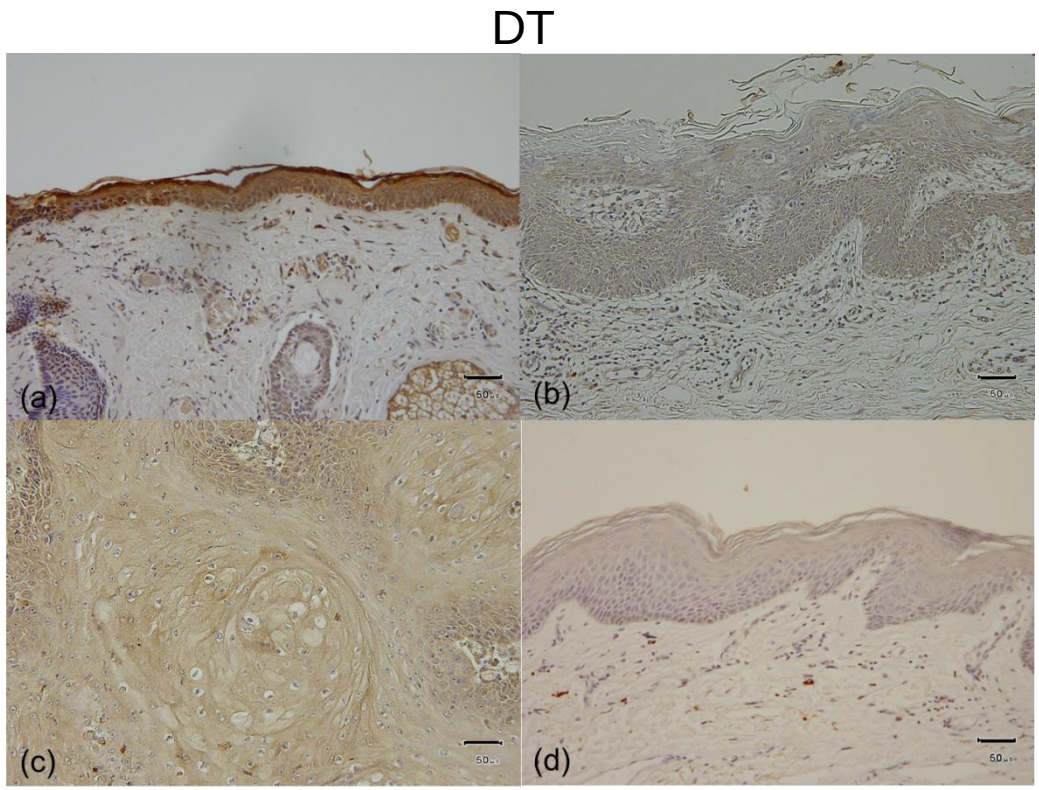
Fig.2A



(**P ≦ 0.01, *P ≦ 0.05)

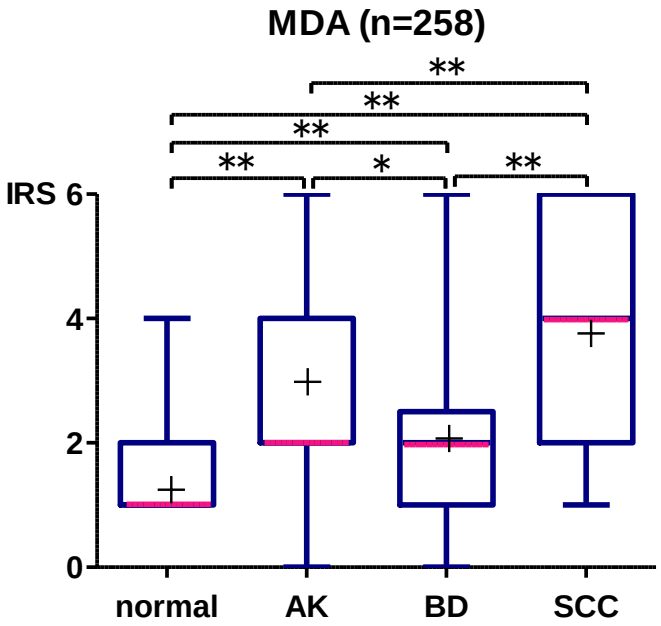
(＋： median)

Fig.2B



(a) AK 3×2	(b) BD 1×2
(c) SCC 2×2	(d) normal 1×2

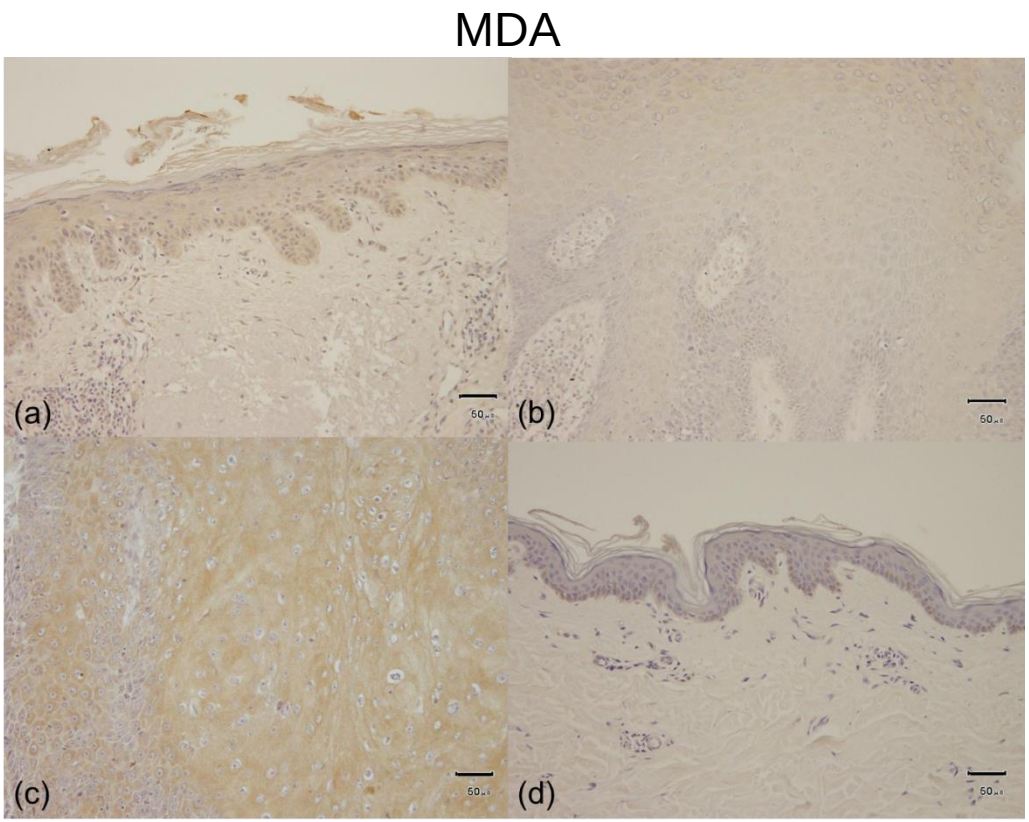
Fig.3A



(**P ≦ 0.01, *P ≦ 0.05)

(＋： median)

Fig.3B



(a) AK 2×2	(b) BD 1×2
(c) SCC 3×2	(d) normal 1×2