iNOS-dependent DNA damage in patients with malignant fibrous histiocytoma in relation to prognosis

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Malignant fibrous histiocytoma (MFH) is one of the most common soft tissue sarcomas. MFH has been proposed to be a lesion accompanied with inflammatory responses. During chronic inflammation, reactive nitrogen and oxygen species generated from inflammatory cells are considered to participate in carcinogenesis by causing DNA damage. 8-nitroguanine is a mutagenic nitrative DNA lesion formed during chronic inflammation. We examined whether nitrative DNA damage is related to the prognosis of MFH patients. We performed immunohistochemical analyses to examine the distribution of DNA damage and the expression of inflammation-related molecules including inducible nitric oxide synthase (iNOS), nuclear factor-κB (NF-κB), and cyclooxygenase-2 (COX-2) in clinical specimens from 25 patients with MFH. We also analyzed the correlation of DNA damage or the expression of these genes with the prognosis of MFH patients. Immunohistochemical staining revealed that the formation of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an oxidative DNA lesion, occurred to a much greater extent in MFH tissue specimens from deceased patients than in live patients. iNOS, NF-κB and COX-2 were colocalized with 8-nitroguanine in MFH tissues. It is noteworthy that the statistical analysis using the Kaplan-Meier method demonstrated strong 8-nitroguanine staining to be associated with a poor prognosis. In conclusion, 8-nitroguanine appears to participate in not only the initiation and promotion of MFH, but also in the progression of MFH, and could therefore be used as a promising biomarker to evaluate the prognosis of cancer patients. (Cancer Sci 2007; 98: 163–168)

Malignant fibrous histiocytoma has been regarded as one of the most common soft tissue sarcomas occurring in adult patients,[1] and it occurs most frequently in the extremities, trunk and retroperitoneum.[2] MFH is classified into four subtypes including storiform-pleomorphic (60–70%), myxoid (10–20%), giant cell (approximately 10%) and inflammatory (relatively rare).[3] MFH has been proposed to be a lesion accompanied with inflammatory responses. The expression of cytokines in a certain type of MFH (inflammatory MFH) may account for the local inflammatory infiltrate and the aggressive nature observed in these malignant cells.[4] In the early phases of experimentally induced rat sarcoma, an inflammatory reaction characterized by an infiltration of lymphocytes, monocytes and macrophages was observed.[5] It has been hypothesized that many malignancies arise from areas of inflammation.[6,7] Moreover, chronic inflammation has also been proposed to contribute to the development of various cancers.[6,7] These findings led us to an idea that the inflammatory responses thus play a role in the pathogenesis of MFH.

During chronic inflammation, RNS and ROS are generated from inflammatory and epithelial cells, and therefore are considered to play a key role in both carcinogenesis and tumor progression.[8,9] ROS can induce the formation of 8-oxod), an indicator of oxidative DNA damage.[10–12] Nitric oxide can mediate the formation of 8-nitroguanine, a marker of nitrative DNA damage,[13] which has been proposed to account for inflammation-mediated carcinogenesis as a potentially mutagenic lesion.[9,14] In MFH patients, the tumor tissues have been reported to have mutations in proto-oncogenes and tumor suppressor genes.[15–17] We have previously performed clinical studies to examine the formation of 8-nitroguanine and 8-oxodG in patients with various inflammatory diseases.[18,19] We have discovered that these DNA lesions were specifically formed at sites of carcinogenesis under various inflammatory conditions.[20–24] 8-nitroguanine was apparently formed in gastric gland epithelial cells of patients with H. pylori infection[20] and in hepatocytes of patients with chronic hepatitis C.[21] Furthermore, we recently performed a molecular epidemiological study which suggested that the formation of 8-nitroguanine is possibly associated with a poor prognosis in cancer patients.[22] In MFH patients, we demonstrated the prognostic significance of the expression of bone morphogenetic protein-2.[25] However, the role of nitrative DNA damage in carcinogenesis in soft tissues remains to be clarified.

In this study, we obtained surgical specimens from MFH patients, and performed a double immunofluorescent staining procedure to examine the formation of 8-nitroguanine and 8-oxodG. Moreover, we examined the expression of iNOS, NF-κB and COX-2 in sarcomas of MFH patients by immunohistochemistry. NF-κB is a key player in inflammation which regulates the expression of various genes involved in controlling inflammatory response such as proinflammatory mediators, iNOS and COX-2.[26,27] To investigate the prognostic factors of cancer patients, we analyzed the relation of 8-nitroguanine formation and the expression of inflammation-related molecules with the survival rate of MFH patients.

Materials and Methods

Subjects. MFH patients undergoing surgical resections from 1990 to 2004 at the Department of Orthopaedic Surgery, Mie University Graduate School of Medicine, Japan, participated in this study. This study was approved by the Ethics Committee of Mie University Graduate School of Medicine. The gross appearance of surgical specimens obtained from 25 patients was classified as Stage IIa (1 patient), Stage IIb (2 patients), Stage III (17 patients) and Stage IV (5 patients) at the original diagnosis

(Table 1). The International Union Against Cancer TNM classification and the staging system by the American Joint Committee on Cancer (AJCC) were used for tumor assessment.

Specimen collection and storage. MFH cases were diagnosed by physicians based on the clinicopathological findings. Surgical specimens were immediately fixed in 20% neutralized formalin buffer solution at room temperature, and then, paraffin blocks were made.

Immunohistological staining. Immunohistochemical staining was performed using the LSAB (labeled streptavidin-biotin) method; 3-μm sections were cut using a microtome (MICROM GmbH, Type HM400R, Walldorf, Germany) and incubated overnight at 55°C. These sections were deparaffinized in xylene and rehydrated in descending gradations of ethanol. To enhance the immunostaining, the sections were placed in citrate buffer (pH 6) and autoclaved at 120°C for 10 min for antigen unmasking. After gradually quenching at room temperature, they were washed by PBS and immersed in APK buffer solution (Ventana Medical Systems, Inc., Tucson, AZ, USA). The sections were then placed on a NexES IHC automated immunostainer (Ventana Medical Systems, Inc.). We produced highly sensitive and specific anti-8-nitroguanine rabbit polyclonal antibody as described previously,[28] and used as primary antibody at a concentration of 2 μg/mL. Other primary antibodies were also employed and their working dilutions were as follows: anti-8-oxodG mouse monoclonal antibody (1:100, Japan Institute for the Control of Aging, Fukuroi, Japan), anti-NF-κB p65 rabbit polyclonal antibody (1:1000, Abcam, Cambridge, UK), anti-iNOS rabbit polyclonal antibody (1:300, Calbiochem-Novabiochem, Darmstadt, Germany) and anti-COX-2 rabbit polyclonal antibody (1:300, Oxford Biomedical Research, Oxford, MI, USA). In a NexES IHC i-VIEW DAB KIT, Bluing Reagent and hematoxylin (Ventana Medical Systems) were employed. As secondary antibodies, anti-mouse IgG, anti-mouse IgM and anti-rabbit IgG antibodies (Ventana Medical Systems) were used. Then, we performed quantitative analysis for immunoreactivities of DNA lesions and inflammation-related molecules by measuring the staining rates using Lumina Vision software (version 1.11, Mitani Shoji Corporation, Japan).

To examine the colocalization of 8-nitroguanine and the expression of inflammation-related molecules, we performed double immunofluorescence technique. Deparaffinized sections were washed in PBS and then immersed in PBS including 0.1% Triton-X for 30 min. Thereafter, they were again washed in PBS. After autoclaving in citrate buffer (pH 6) at 120°C for 10 min, the sections were quenched at room temperature. Next, the sections were blocked with 1% skim milk for 30 min and treated with rabbit polyclonal anti-8-nitroguanine antibody and mouse monoclonal antibody (anti-8-oxodG, anti-NF-κB p65, anti-iNOS [1:300; Sigma, St. Louis, MO, USA] or anti-COX-2 [1:50, Cayman Chemical, Ann Arbor, MI, USA] antibody) overnight at room temperature. After washing in PBS, they were incubated for 3 h with Alexa 594-labeled goat antibody against rabbit IgG and Alexa 488-labeled goat antibody against mouse IgG (Molecular Probes, Eugene, OR, USA, 1:400 each). The stained sections were examined under both an inverted microscope and a fluorescence microscope (BX50F-3, Olympus Optical Co. Ltd., Tokyo, Japan).

Histopathological study. A histopathological study was performed by hematoxylin and eosin staining in paraffin sections by the standard method. The deparaffinized sections were washed in running tap water for 5 min, and then the slides were immersed in Mayer's Hematoxylin (Merck Sharp & Dohme, Whitehouse Station, NJ, USA) for 2–3 min. After washing in running tap water for 5 min, the slides were immersed in 70% alcohol for 2 min, in eosin alcohol for 1–2 min, and in 90% alcohol for a period and then were dehydrated.

Table 1. Clinicopathological data of the malignant fibrous histiocytoma patients

No.	Age	Sex	AJCC stage	Location	Size(cm)	Recurrence	Metastasis	Histological type	Follow-up duration (month)	Prognosis
1	47	F	IV	Buttock	6 × 4 × 3	+	Lung, skin, thigh, pleura	Storiform-pleomorphic	45	DOD
2	50	F	III	Buttock	6 × 5 × 5	+	Lung	Storiform-pleomorphic	68	NED
3	73	F	III	Chest wall	12 × 10 × 6	–	Intrathoracic	Storiform	33	DOD
4	76	F	III	Back	8 × 4 × 2	+	Axillary lymph node	Storiform	24	DOD
5	64	F	III	Thigh	12 × 4 × 4	+	–	Storiform	102	NED
6	54	M	III	Back	10 × 8 × 6	–	–	Storiform	15	AWD
7	75	M	IV	Buttock	18 × 11 × 5	+	Lung, inguina	Storiform	8	DOD
8	73	M	IV	Thigh	20 × 15 × 13	+	Lung	Storiform	9	DOD
9	81	F	III	Thigh	19 × 13 × 10	–	–	Storiform	38	CDF
10	69	F	III	Thigh	16 × 10 × 8	+	–	Storiform	64	NED
11	75	M	III	Thigh	5 × 5 × 4	+	Liver	Storiform	42	NED
12	66	M	III	Thigh	10 × 10 × 8	+	Lung	Pleomorphic	12	DOD
13	49	M	IV	Thigh	15 × 10 × 10	+	Lung	Pleomorphic	15	DOD
14	67	M	III	Upper arm	16 × 10 × 10	–	Lung	Pleomorphic	12	DOD
15	57	F	III	Thigh	22 × 16 × 10	–	–	Pleomorphic	87	CDF
16	69	M	III	Upper arm	13 × 8 × 7	–	–	Pleomorphic	33	CDF
17	46	F	IIb	Knee	6 × 5 × 2	–	–	Pleomorphic	52	CDF
18	42	M	III	Lower leg	9 × 8 × 6	–	–	Myxoid	40	CDF
19	70	F	IIb	Thigh	11 × 11 × 4	–	Lung	Myxoid	156	NED
20	77	M	IIa	Upper arm	10 × 9 × 4	+	Axillary lymph node	Myxoid	78	DOD
21	69	F	III	Thigh	12 × 9 × 6	–	Intrapelvic	Myxoid	29	DOD
22	27	M	IV	Thigh	20 × 20 × 5	–	Lung	Myxoid	5	DOD
23	85	F	III	Back	15 × 13 × 8	+	Lung	Myxoid	4	DOD
24	54	F	III	Thigh	11 × 10 × 4	–	–	Myxoid	41	CDF
25	53	M	III	Retroperitoneum	18 × 13 × 11	–	–	Myxoid	43	CDF

AJCC, American Joint Committee on Cancer; DOD, dead of disease; CDF, continuous disease free; NED, no evidence of disease; AWD, alive with disease.

Statistical analysis. The patient samples were categorized into six subgroups according to the staining rates (<7.5%, 7.5–15.0%, 15.0–22.5%, 22.5–30.0%, 30.0–37.5% and >37.5%), evaluated as described above, and then statistical differences of the immunoreactivities between deceased and living patients were analyzed by the χ^2-test. Spearman's and Pearson's rank correlation coefficients served to analyze the correlations for qualitative and quantitative data, respectively. Survival between the two subgroups, high-grade (staining rates; 15% or more) and low-grade (less than 15%) groups, was compared using the life-table method of Kaplan-Meier, and then statistically analyzed by the generalized Wilcoxon test. P-values of less than 0.05 were considered to be statistically significant.

Results

Clinical data of MFH patients. The 25 MFH patients undergoing surgical resections comprised 12 male and 13 female patients, ranging in age from 27 to 85 years (mean ± SD, 62.7 ± 14.1 years). Survival data were available for all patients. The follow-up duration ranged from 4 to 156 months (median, 46 months). These specimens were verified by histopathological studies and the histological subtypes were categorized into storiform-pleomorphic (2 patients), storiform (9 patients), pleomorphic (6 patients) and myxoid (8 patients) by two independent researchers according to Enzinger and Weiss's Soft Tissue Tumors criteria.[1] One of the two patients demonstrating a storiform-pleomorphic subtype, 4/9 patients with a storiform subtype, 3/6 with a pleomorphic subtype and 4/8 with a myxoid subtype died. There was no significant difference in the prognosis among histological subtypes. All tumors measured more than 6 cm diameter in size. The tumors were located in the thigh (12 patients), limb (4 patients), buttock (3 patients), back (3 patients), chest wall (1 patient), knee (1 patient) and retroperitoneum (1 patient). Recurrences were found in 12 cases (48%) and distant metastases were found in 15 cases (60%). Among these 15 cases, 10 had lung metastasis. The prognosis was classified as follows: DOD, death of disease (12 patients); NED, no evidence of disease (5 patients); CDF, continuous disease free (7 patients); AWD, alive with disease (1 patient). The clinical data of these patients are summarized in Table 1.

Histopathological findings and 8-nitroguaine formation in tumor tissues of MFH patients. Figure 1 shows the histopathological findings and 8-Nitroguanine formation in MFH patients. The patients with storiform, pleomorphic and myxoid types showed such characteristic histological features as described previously.[1,2] 8-nitroguanine formation was observed in the nuclei of the tumor cells and inflammatory cells within the MFH tissue specimens. The immunoreactivity of 8-nitroguanine was observed to a significantly greater extent in the patients who died due to any histological subtype (storiform, pleomorphic and myxoid) than in the living patients (Fig. 1 and Table 2).

DNA damage and expression of inflammation-related molecules in tumor tissues of MFH patients. Figure 2 shows DNA damage and the expression of inflammation-related molecules in tumor tissues and adjacent non-tumor tissues of MFH patients. The formation of 8-nitroguanine and 8-oxodG was clearly observed in the nucleus of tumor cells. iNOS and COX-2 proteins were localized in the cytoplasm of tumor cells. The expression of NF-κB was more evidently detected in the nucleus compared with the cytoplasm of tumor cells. On the other hand, DNA damage and the expression of these inflammation-related genes were not observed in adjacent non-tumor tissues (Fig. 2). No immuno-reactivity was observed in tumor tissues when the primary antibody was omitted (data not shown). Figure 3 shows the colocalization of 8-nitroguanine (red) with 8-oxodG, NF-κB, iNOS and COX-2 (green), as evaluated by the double immuno-fluorescence technique. 8-Nitroguanine and 8-oxodG were
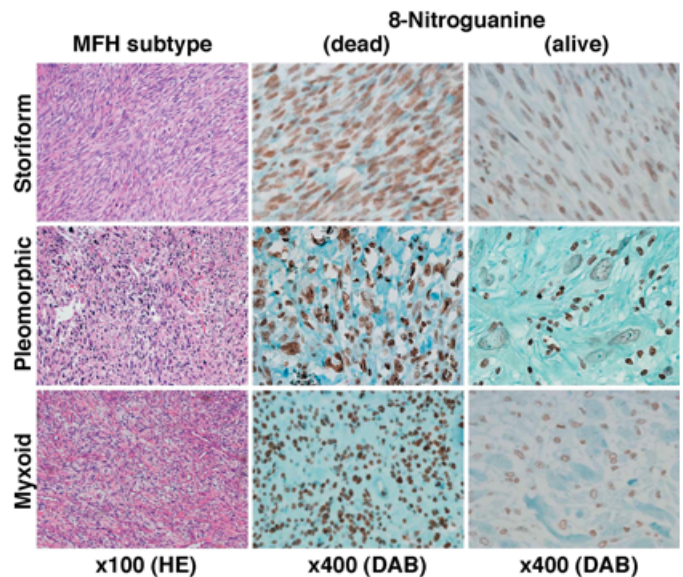

Fig. 1. Histopathological findings and 8-nitroguanine formation in malignant fibrous histiocytoma (MFH) patients. 8-nitroguanine formation is observed in the nuclei of tumor cells and inflammatory cells within MFH tissue specimens. The immunoreactivity of 8-nitroguanine is found to be significantly greater in the patients who died than in the living subjects regardless of any histologic subtype.

colocalized in the same tumor cells. The expression of iNOS and COX-2 was observed in the cytoplasm in most 8-nitroguanine-positive tumor cells. NF-κB expression was detected mainly in the nucleus and colocalized with 8-nitroguanine. In addition, the immunoreactivities of 8-nitroguanine, 8-oxodG, NF-κB and COX-2 were also observed in giant cells and inflammatory cells in MFH tissues.

Influences of DNA damage and inflammation-related molecules on survival of MFH patients. We statistically analyzed the difference of DNA damage and the expression of inflammation-related molecules between deceased and living patients as shown in Table 2. Immunoreactivity of 8-nitroguanine was significantly stronger in the tumor tissue of the deceased patients than in those of the living patients ($P = 0.0003$). Similarly, the immuno-reactivities of 8-oxodG ($P = 0.0001$), iNOS ($P = 0.0163$), NF-κB ($P = 0.0092$) and COX-2 ($P = 0.0007$) were significantly stronger in the patients who died (Table 2).

We also performed generalized Wilcoxon test using the Kaplan-Meier method to evaluate the effects of DNA damage and inflammation-related molecules on prognosis of MFH patients as shown in Figure 4. MFH patients with high-grade levels (staining rate; ≥15%) of 8-nitroguanine had a significantly shorter survival ($P = 0.0002$) than those with low-grade levels (<15%) (Fig. 4). In addition, the patients with high-grade levels of 8-oxodG ($P = 0.0002$), iNOS ($P = 0.0278$), COX-2 ($P = 0.0007$) and nuclear NF-κB ($P = 0.0080$) exhibited a significantly poorer prognosis in comparison to those with low-grade levels (Fig. 4). These results suggest that the formation of DNA lesions and the expression of these molecules are significantly associated with poor survival. Moreover, MFH patients with metastasis also had a significantly shorter survival ($P = 0.0005$) than those without metastasis (Fig. 4).

Correlation of the 8-nitroguanine formation with metastasis, recurrence and the expression of inflammation-related molecules. The prognosis for MFH patients (deceased or alive) showed a much more remarkable correlation with metastasis ($R = 0.852$, $P = 0.0000$) than with recurrence ($R = 0.4423$, $P = 0.0268$). 8-nitroguanine formation was strongly correlated with metastasis

Table 2. Difference in DNA damage and the expression of imflammation-related molecules between deceased and living malignant fibrous histiocytoma patients

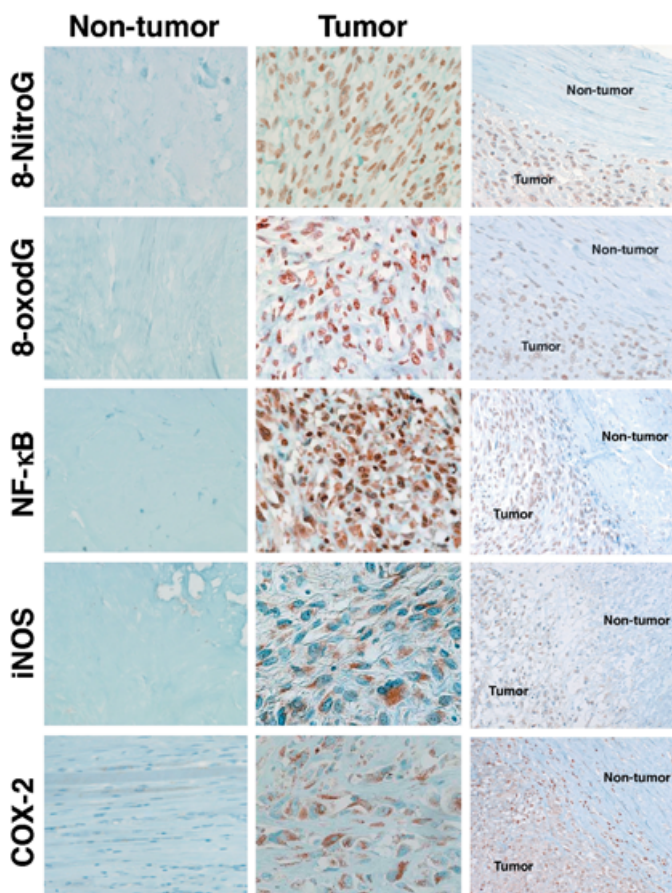| Staining rate (%)	8-Nitroguanine		8-oxodG		iNOS		NF-κB (nucleus)		COX-2	
	Alive (13)	Dead (12)	Alive (13)	Dead (12)	Alive (13)	Dead (12)	Alive (13)	Dead (12)	Alive (13)	Dead (12)
					Number of patients(%)					
0.0–7.5	5	0	11	1	5	1	7	1	8	0
7.5–15.0	8	1	2	0	4	0	4	1	4	1
15.0–22.5	0	7	0	6	3	3	2	3	0	0
22.5–30.0	0	2	0	5	1	7	0	4	1	2
30.0–37.5	0	2	0	0	0	1	0	3	0	7
>37.5	0	0	0	0	0	0	0	0	0	2
P-value	P = 0.0003		P = 0.0001		P = 0.0163		P = 0.0092		P = 0.0007	

Fig. 2. DNA damage and the expression of nuclear factor-κB (NF-κB), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in malignant fibrous histiocytoma tissues. The formation of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is observed in the nucleus of tumor cells. iNOS and COX-2 proteins are expressed in the cytoplasm in most tumor cells. NF-κB is more clearly detected in the nucleus than in the cytoplasm of tumor cells. Little or no immunoreactivity of these DNA lesions and proteins was observed in adjacent non-tumor tissues. Magnification: ×400 (left and center columns) and ×200 (right column).
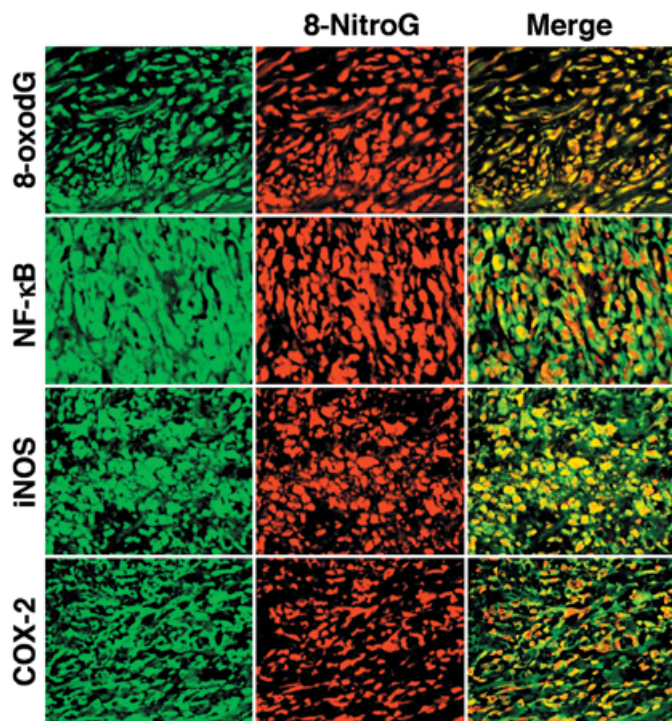

Fig. 3. Colocalization of 8-nitroguanine with 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), nuclear factor-κB (NF-κB), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). 8-Nitroguanine and 8-oxodG are colocalized in the same tumor cells. iNOS and COX-2 are expressed in the cytoplasm in most 8-nitroguanine-positive tumor cells. NF-κB is colocalized with 8-nitroguanine in the nucleus. Magnification: ×400.

$(R = 0.7040, P = 0.0001)$. 8-Nitroguanine formation was correlated with the formation of 8-oxodG $(R = 0.8207, P = 0.0000)$ and expression of iNOS $(R = 0.5846, P = 0.0022)$, NF-κB $(R = 0.6276, P = 0.0008)$ and COX-2 $(R = 0.7392, P = 0.0000)$. NF-κB expression was correlated with the expression of iNOS $(R = 0.8200, P = 0.0000)$ and COX-2 $(R = 0.7254, P = 0.0000)$ in MFH tissues.

Discussion

Chronic inflammation has been proposed to contribute to the onset and development of various cancers.[6,7] ROS and RNS are considered to play an important role in the pathogenesis of inflammation-related cancer due to the fact that they cause DNA damage.[8,9] We have previously demonstrated that 8-nitroguanine is formed at the sites of carcinogenesis in humans and experimental animals with bacterial, viral and parasite infection.[18–21,29,30] Based on the findings of our clinical studies, we proposed the hypothesis that 8-nitroguanine formation contributes to tumor initiation and promotion.

In this study, to investigate whether 8-nitroguanine can be a prognostic factor of inflammation-related cancer, we examined the formation of these DNA lesions in patients with MFH. In all subtypes of MFH, the infiltration of inflammatory cells was
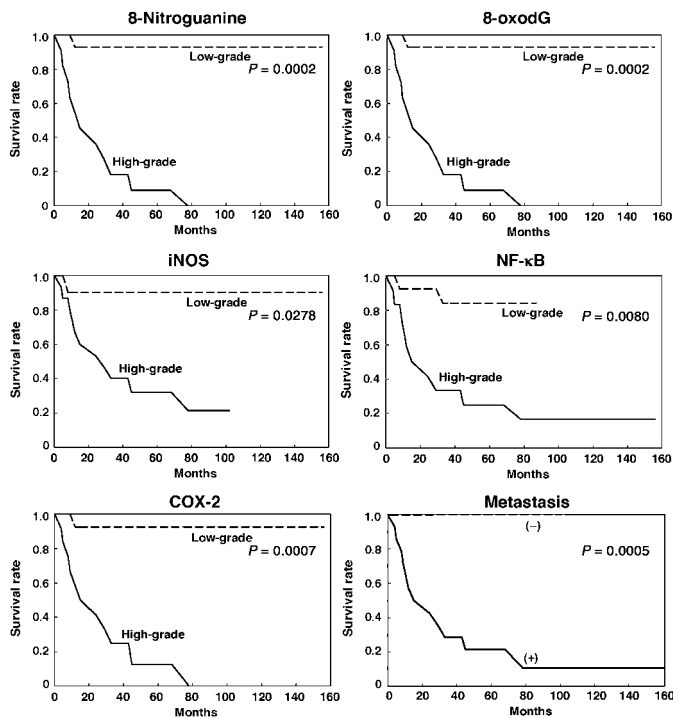
Fig. 4. Effects of DNA damage and inflammation-related molecules on survival of malignant fibrous histiocytoma patients (Kaplan–Meier method). *Solid line*, patients with high-grade levels of DNA lesion (8-nitroguanine or 8-oxo-7,8-dihydro-2′-deoxyguanosine [8-oxodG]) or inflammation-related molecules (nuclear factor-κB [NF-κB], inducible nitric oxide synthase [iNOS] or cyclooxygenase-2 [COX-2]). *Broken line*, patients with low-grade levels. In the case of metastases, *solid and broken lines* represent patients with (+) and without (–) metastases, respectively.

observed in tumor tissue specimens of MFH patients and our findings were consistent with those of previous studies.[2] Both 8-nitroguanine and 8-oxodG formed in sarcoma tissues to a much greater extent in deceased patients than in living subjects. Although among 13 living patients, 5 and 11 cases showed low staining rate (0–7.5%) of 8-nitroguanine and 8-oxoguanine, respectively, their immunoreactivity was higher than that of non-tumor tissues where no immunoreactivity was observed. This result suggests that nitrative and oxidative DNA damage participates in not only initiation but also progression of MFH. Moreover, we demonstrated for the first time that intensive 8-nitroguanine formation in MFH patients is associated with a poor prognosis. Relevantly, we recently performed a molecular epidemiological study, which suggested that 8-nitroguanine formation is possibly associated with a poor prognosis in cholangiocarcinoma patients.[22] These findings raise the hypothesis that 8-nitroguanine formation participates in tumor progression in addition to tumor initiation and promotion. This hypothesis is supported by our recent study showing that subcutaneous implantation of a plastic plate induced soft tissue sarcoma in experimental animals via 8-nitroguanine formation.[31] Moreover, 8-nitroguanine may be used as a better biomarker to predict the potential risk of tumor progression than histological features, because no significant difference was observed in the prognosis between each subtype of MFH.

We also examined the expression of inflammation-related genes in MFH patients in relation to 8-nitroguanine formation and the prognosis of these patients. NF-κB is considered to be a key player in inflammation since it regulates the expression of various genes involved in controlling inflammatory response, including iNOS and COX-2 expression.[26] In addition, NF-κB functions as a tumor promoter in inflammation-associated

cancer.[32] In this study, 8-nitroguanine was colocalized with iNOS and NF-κB in tumor cells of MFH patients. NF-κB expression in the nucleus of tumor cells was closely correlated with iNOS expression in MFH patients. These results lead to the hypothesis that 8-nitroguanine formation is dependent on iNOS expression mediated by NF-κB activation. This is the first study to propose the molecular mechanism of inflammation-related carcinogenesis in soft tissues. The inhibition of NF-κB by the expression of its inhibitory molecule IκBα decreased the viability of human sarcoma cells.[33] iNOS expression was observed in soft tissue sarcoma cells and the treatment with an iNOS inhibitor reduced the growth of the sarcomas in an animal model.[34] These studies support the involvement of NF-κB-mediated iNOS expression in the pathogenesis of MFH. Furthermore, we clearly demonstrated COX-2 expression in MFH, which was colocalized with 8-nitroguanine formation and was significantly associated with a poor prognosis in MFH patients. A selective COX-2 inhibitor has also been reported to inhibit cell growth in a human MFH cell line.[35] In addition, a clinical analysis demonstrated that COX-2 expression was noted in a large part of MFHs, but not in benign fibrohistiocytic tumors, while COX-2-positive MFH showed a poorer survival than negative ones.[36] COX-2 expression is associated with tumor growth and neovascularity in a variety of malignancies.[36–38] Based on our results, it is therefore reasonable to consider that in MFH patients, both NF-κB expression and its nuclear translocation occur, thereby leading to iNOS expression and 8-nitorguanine formation. COX-2 expression appears to be induced by a similar mechanism, thus participating in additional inflammatory responses, and also resulting in tumor progression. NO can stimulate tumor growth and metastasis by promoting the migratory, invasive, and angiogenic abilities of tumor cells, which may also be triggered by the activation of COX-2.[39] MFH has an aggressive behavior and a highly metastatic potential to other organs which can thus result in the eventual death of such patients with soft-tissue sarcomas.[1] Most metastases occur in the lung[2] and consistently, in this study, 10 cases had lung metastasis among the 15 patients with distant metastases. Interestingly, 8-nitroguanine formation was correlated with distant metastases in MFH patients, thus suggesting that the formation of this DNA lesion participates in tumor progression and metastasis. These findings support the hypothesis that DNA damage mediated by inflammatory responses contributes to tumor progression in soft tissues.

ROS and RNS are considered to be involved in the pathogenesis of inflammation-related carcinogenesis through DNA damage.[8,9] These reactive species can induce 8-oxodG formation thus leading to $G \rightarrow T$ transversions.[40,41] On the other hand, RNS generated during inflammation can cause 8-nitroguanine formation. 8-Nitroguanine formed in DNA is chemically unstable, and thus can be spontaneously released, thereby resulting in the formation of an apurinic site.[13] The apurinic site can form a pair with adenine during DNA synthesis, thus resulting in $G \rightarrow T$ transversions[42] Translesion DNA synthesis past an apurinic site mediated by DNA polymerase ζ may contribute to point mutations.[43] Therefore, 8-nitroguanine is a potentially mutagenic DNA lesion as well as 8-oxodG. Gene mutations in tumor tissue specimens from MFH patients have been reported. In MFH patients, the tumor tissues contained $G \rightarrow T$ transversions in the H-*ras* proto-oncogene[15] and an exon of the *p53* tumor suppressor gene.[16] These clinical studies support our findings, which show the formation of mutagenic DNA lesions to be mediated by the inflammatory responses that participate in the carcinogenesis of soft tissues. ROS are generated from multiple sources, including carcinogenic chemicals and their metabolites, and electron transport chain in mitochondria in addition to inflammation. On the other hand, NO is generated specifically during inflammation in inflammatory and epithelial cells. Therefore, 8-nitroguanine is formed mainly under inflammatory

conditions and would play a substantial role in inflammation-related carcinogenesis including MFH, although 8-oxodG can be generated during inflammation.

We have previously reported that various infectious and inflammatory diseases induce the formation of 8-oxodG and 8-nitroguanine and these DNA lesions would participate in the initiation and/or promotion of carcinogenesis.[18,19] In this study, we demonstrated for the first time that the formation of 8-nitroguanine and the expression of the molecules involved in DNA damage are significantly associated with a poor prognosis for cancer patients. The progression of a large majority of human and experimental tumors therefore seems to be stimulated by NO which results from the activation of iNOS.[39] These findings raise a new hypothesis that 8-nitroguanine contributes to tumor progression in addition to initiation and/or promotion. Therefore, the formation of DNA lesions mediated by inflammatory responses could contribute to multiple steps of carcinogenesis, thus leading to tumor invasion and metastasis. In conclusion, 8-nitroguanine is therefore considered to be a potential biomarker to evaluate the potential risk and prognosis of patients with inflammation-related cancer.

Acknowledgments

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