Immunohistochemical Analysis for Basal Activation of NF-κB in Acral Lentiginous Melanoma

Jae Hoon Jung, M.D., Min Geol Lee, M.D.*, Sang-Ho Cho, M.D.**, You Chan Kim, M.D., Hyang Joon Park, M.D., Yong Woo Cinn, M.D., Kye Young Lee, M.D.***

*Department of Dermatology and Internal Medicine***, College of Medicine, Dankook University, Cheonan, Korea; Department of Dermatology* and Pathology**, College of Medicine, Yonsei University, Seoul, Korea*

Background **: Spontaneous basal activation that might be related to survival mechanism of tumor cells by allowing them to escape from apoptosis has been proven in some tumor cells, but it has not been evaluated in malignant melanoma tissue.**

Objective **: The purpose of this study was to evaluate basal activation of NF-κB using immunohistochemical analysis and demonstrate its clinical significance in cutaneous malignant melanoma tissue.**

Materials and Methods **: Twenty-five cases of acral lentiginous melanoma(ALM) from 20 patients were selected. Immunohistochemical analysis was performed to detect nuclear localization of classic NF-κB heterodimer, p50 and p65 in the formalin-fixed, paraffin-embedded tissues.**

Results **: In about 50% of cases, nuclear expression of NF-κB heterdimer, p50 and p65 was detected, 12(48%) and 13(52%) of 25 cases of ALM, respectively. However, the nuclear expressing of p50 and p65 was not significant for tumor thickness or level of invasion in ALM.**

Conclusion **: We demonstrated basal NF-κB activation in malignant melanoma, but we need to research further to demonstrate its clinical significance.**

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Key Words : Acral lentiginous melanoma, Basal NF-κB activation, p65, p50, Immunohistochemistry

Nuclear factor-κ B (NF-κ B) is a transcription factor that plays an important role in the inflammatory process and immune reaction[1]. It was first identified as a B-cell nuclear factor and given its name on the basis of its ability to bind to an intronic enhancer of the immunoglobulin κ -light chain gene. Presently, five mammalian NF-κB family members have been identified and cloned. These include NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65(RelA), RelB, and c-Rel. A characteristic feature of NF-κB is that all of the family members share a highly conserved Rel homology domain. The most abundant activated form of NF-κB is a heterodimer composed of a p50 or p52 subunit and a p65 subunit. Other dimeric complexes have also been detected in some cell types under certain culture conditions[2]. In unstimulated cells, NF-κB is in the cytoplasm in an inactive form bound to an inhibitory protein known as IκB. Stimulation, including cytokines, oxidant free radicals, and bacterial or viral products, leads to rapid phosphorylation, ubiquitinylation, and ultimately proteolytic degradation of IκB. NF-κ B which frees from IκB translocates to the nucleus

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Reprint request to : Kye-Young Lee, M.D., Department of Internal Medicine, Dankook University College of Medicine, San 29, Anseo-dong, Cheonan 330-714, Korea
Tel. (041)550-3916, Fax. (041)556-3256
E-mail : kyleemd@anseo.dankook.ac.kr

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and activates the transcription of its target genes involved in inflammatory and immune responses[2-6]. NF-κB also has an anti-apoptotic effect and it is stimulated by tumor necrosis factor-α, ionizing radiation or the chemotherapeutic agent, daunorubicin[7-11]. Recently, it has been found that NF-κB is basally activated without any stimuli in some solid tumors and this finding suggests that the basal nuclear expression of NF-κB may contribute to carcinogenesis through anti-apoptotic effect[12-14]. However, the expression of NF-κ B in malignant melanoma tissue has not been studied. To investigate the basal activation of NF-κ B in malignant melanoma, we examined immunohistochemical findings of acral lentinginous melanoma (ALM) using NF-κB p50 and p65 antibodies. We also analyzed the relation between basal nuclear expression of p50 and p65, tumor thickness and level of invasion in malignant melanoma.

MATERIALS AND METHODS

Subjects

Twenty-five specimens of ALM from 20 patients at Yonsei University Hospital, Seoul, Korea from 1990 to 1998 were selected for study. These included 23 primary and 2 metastatic ALMs. Tumor thickness and level of invasion were measured by Breslow microstage and Clark classification, respectively (Table 1).

Immunohistochemistry

Immunohistochemical staining for p50 (Santa Cruz biotechnology, CA, USA) and p65 (Santa Cruz biotechnology, CA, USA) was performed on formalin-fixed, paraffin-embedded tissue. For preparation, sections were deparaffinized in xylene, using 3 changes, 5 minutes each, and then rehydrated with 100%, 90%, 80%, 70% ethanol, using 2 changes, 2

Table 1. Clinicopathologic findings of 25 cases of acral lentiginous melanoma.

Case	Sex/Age	Location	Type	Breslow microstage	Clark level
1	M/60	rt. great toe	primary	4	Ⅳ
2	M/72	rt. foot	metastatic	4	Ⅴ
3	M/65	rt. foot	primary	4	Ⅲ
4	F/56	lt. heel	primary	2	Ⅲ
5	F/57	lt. heel	primary	2	Ⅱ
6	F/66	lt. heel	primary	4	Ⅴ
7		lt. heel	primary	4	Ⅴ
8	F/64	lt. heel	primary	4	Ⅴ
9	M/71	lt. heel	primary	4	Ⅳ
10	M/65	lt. heel	primary	3	Ⅲ
11	M/62	lt. heel	primary	4	Ⅴ
12	F/58	rt. heel	primary	2	Ⅱ
13	M/74	rt. heel	primary	4	Ⅳ
14	F/52	rt. heel	primary	4	Ⅳ
15	M/78	rt. heel	primary	2	Ⅱ
16		rt. heel	primary	3	Ⅲ
17	M/76	sole	primary	4	Ⅳ
18	M/72	lt. sole	primary	3	Ⅲ
19		lt. sole	primary	4	Ⅳ
20	M/51	rt. sole	primary	4	Ⅳ
21		rt. sole	primary	4	Ⅳ
22	F/73	thumb	primary	1	Ⅰ
23	M/75	rt. thumb	metastatic	4	Ⅳ
24	M/47	rt. thumb	primary	4	Ⅳ
25		rt. thumb	primary	4	Ⅳ

rt: right, lt: left

minutes each. Antigen determinants masked by paraffin-embedding were unmasked by enzymatic digestion. The microwave antigen retrieval method with a 0.01M sodium citrate buffer, pH 6.0 was performed to expose antigenic site and reduce background. In the immunoperoxidase procedure, tissue sections were first incubated with the second step antibody, followed by incubation with a streptavidin-horseradish peroxidase complex (universal large volume DAKO LSAB kit, Japan co., Ltd). Staining was accomplished with slides in AEC (3-amino 9-ethylcarbazole) as chromogen and counterstained in mayer hematoxylin.

Staining assessment

p50 and p65 nuclear reactivities were estimated and scored as follows; 0: <10% of nucleus of tumor cells stained, 1: 10~33% of nucleus of tumor cells stained, 2: 34~66% of nucleus of tumor cells stained, 3: >66% of nucleus of tumor cells stained. Cytoplasmic reactivities of tumor cells were scored by the same method to investigate the relationship between the nucleus/cytoplasm (N/C) ratio of NF-κB reactivity with the tumor thickness and with level of invasion. N/C ratio of NF-κB reactivity was scored as follows; 0: <0.1, 1: 0.1~0.25, 2: 0.25~0.5, 3: 0.5~0.75, 4: >0.75.

Statistical analysis

The correlations between p50 and p65 nuclear expressions, tumor thickness and level of invasion were analyzed using Spearman correlation coefficient (SPSS version 9.0). The correlations between N/C ratio of NF-κB reactivity, tumor thickness and level of invasion were also analyzed by same method. Statistical significance was defined as $p<0.05$.

RESULTS

The clinical data is summarized in Table 1. Tumors ware found in 14 men and 6 women (mean age; 68.4). Sites biopsied for tumor involvement included heels (13 cases), feet (3 cases), soles (5 cases), and thumbs (4 cases). Breslow depth of tumor revealed 68% to be thick (>3mm) at the time of excision. Clark's level of the tumor in the majority of the cases (84%) was Ⅲ or more at the time of excision. Results concerning p50 and p65 nuclear expressions and N/C ratios of NF-κB reactivity are given in Table 2. Both the nuclei and cytoplasms of tumor cells stained with p50 in 12 of 25 cases (Fig. 1), and stained with p65 in 13 of 25 cases (Fig. 2). However, there were 23 (92%) and 21 (84%) cases respectively in which p50 and p65 staining was less than 33%. The correlation between basal nuclear expression, N/C ratio of NF-κB reactivity, tumor thickness and level of invasion are summarized in Table 3-1 and Table 3-2. No significant association was found between basal nuclear expression of p50 and p65 with tumor thickness or with level of invasion ($p>0.05$). There was no correlation between N/C ratio of NF-κB reactivity, tumor thickness and level of invasion($p>0.05$). No statistical

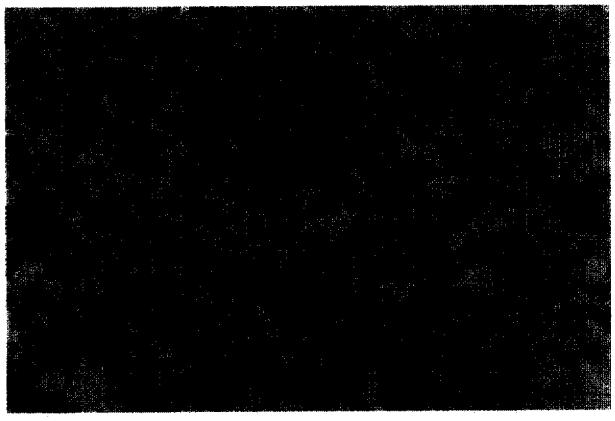

Fig. 1. Immunohistochemical staining with p50 antibodies in the acral lentiginous melanoma. The positive cytoplasmic and nuclear staining of p50 is seen in the tumor cells. (× 200).

Fig. 2. Immunohistochemical staining with p65 antibodies in the acral lentiginous melanoma. The tumor cells are reactive for p65. (× 200).

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significance of clinical parameters including sex, age and location to NF-κB reactivity was found.

DISCUSSION

Apoptosis is the general name for physiologic cell death caused by a genetically encoded program[15,16]. In the dermatologic field, it is also regarded as a fundamental part in epidermal homeostasis and has been detected in some skin tumors[17]. One of the well characterized factors that can inhibit apoptotic signals is bcl-2. Actively proliferating cells express bcl-2, whereas terminally differentiated cells lose bcl-2 expression[16]. Aberrant expression of bcl-2 has been involved in tumor development, and changes in bcl-2 levels have been observed in some skin cancers. In addition to bcl-2, transcription factor NF-κB and other gene family of inhibitors of apoptosis (IAP) including survivin, Hsp 70, Hsp 72 have an anti-apoptotic effect. In normal and neoplastic skin the expression of NF-κB and IAP was investigated[17]. The evidence of a connection between NF-κB and cell death came from studies on 'knockout' mice that lack the 65 kD RelA subunit (also known as p65) of NF-κB as a result of targeted mutation of the RelA gene. These mice died before birth because of massive degeneration of liver cells by apoptosis suggesting that in mice, NF-κB has a protective role during early liver development[18]. Beg et al[7] demonstrated that fibroblasts and macrophages from the RelA deficient mice are sensitized to TNF-α induced cytotoxicity and die within eight hours of exposure to TNF-α, whereas cells from wild-type mice survive.

Table 2. Immunohistochemical results about reactivities of p50 and p65 antibodies.

Case	p50 (nuc.)	p50 (N/C)	p65 (nuc.)	p65 (N/C)
1	1	1	1	2
2	1	1	1	2
3	1	2	2	3
4	1	2	0	0
5	0	0	3	4
6	0	0	0	0
7	0	0	3	4
8	0	0	2	3
9	0	0	0	0
10	0	0	0	0
11	0	0	1	2
13	1	2	1	1
14	1	1	1	3
15	0	0	0	0
16	0	0	1	1
17	1	1	0	0
18	1	2	0	0
19	0	0	0	0
20	1	1	1	2
21	0	0	1	1
22	1	2	0	0
23	0	0	0	0
24	0	0	0	0
25	2	2	0	0

nuc.: nuclear expression, N/C: nuclear/cytoplasmic ratio of NF-κB reactivity
Nuclear expression was scored as follows, 0: <10% of nucleus of tumor cells stained, 1: 10~33% of nucleus of tumor cells stained, 2: 34~66% of nucleus of tumor cells stained, 3: >66% of nucleus of tumor cells stained. N/C ratio of NF-κB reactivity was scored as follows; 0: <0.1, 1: 0.1~0.25, 2: 0.25~0.5, 3: 0.5~0.75, 4: >0.75.

Table 3-1. Statistical evaluation of p50 nuclear expression and N/C ratio of NF-κB reactivity in tumor thickness and level of invasion.

		p50 (nuc.)	p50 (N/C)
Tumor thickness	coefficient	-0.235	-0.303
	probability*	0.258	0.141
Level of invasion	coefficient	-0.254	-0.328
	probability*	0.220	0.109

nuc.: nuclear expression, N/C: nuclear/cytoplasmic ratio of NF-κB reactivity.
* : probabiltiy >0.05 is not significant

Table 3-2. Statistical evaluation of p65 nuclear expression and N/C ratio in tumor thickness and level of invasion.

		p65 (nuc.)	p65 (N/C)
Tumor thickness	coefficient	0.03538	0.3470
	probability*	0.0828	.0892
Level of invasion	coefficient	0.1865	0.1814
	probability*	0.3721	0.3855

nuc.: nuclear expression, N/C: nuclear/cytoplasmic ratio of NF-κB reactivity
* : probabiltiy >0.05 is not significant

However, the susceptibility of RelA deficient cells to TNF-α induced toxicity is reversed following transfection of the cells with the wild-type RelA gene. As a result, they concluded that NF-κB is involved in apoptosis. Recently, Wang et al[19] reported that RelA subunits are basally activated about 67% without any stimulation in the nucleus of pancreatic cancer tissue[19]. Also, basal nuclear expression of NF-κB has been shown in other tumor cell lines including cutaneous T cell lymphoma, B cell lymphoma, lung cancer and cutaneous melanoma[5] but most of these cells were studied in cell culture system, not with tissue specimens from patient. Malignant melanoma is the most serious cancer arising in the skin, and its incidence is increasing more rapidly than any other cancer[19]. Melanoma is almost uniformly resistant to cytotoxic intervention. The biological basis for the general pharmacological resistance of melanoma is not well understood[5], but defects of the cell death program have been described[20]. Meyskens et al[5] measured the level of NF-κB in metastatic melanoma cells and normal melanocytes. The basal DNA-binding activity of NF-κB increased 4-fold compared with that of normal melanocytes. However, Haycock et al6 observed no constitutive level of NF-κB activity in human ocular melanoma cells and melanocytes. To examine the basal nuclear activation of NF-κB in the malignant melanoma tissue, we used an immunohistochemical stain that is useful for localization of intracellular gene expression in the formalin-fixed, paraffin embedded tissue specimen. Antibodies p50 and p65 were used because their heterodimers are the most abundant activated form of NF-κB. Nuclear reactivity was found in 12 (48%) with anti p50 and 13 (52%) cases with anti p65 of our twenty-five specimens of ALM from 20 patients. In addition, we also analyzed the N/C ratio of NF-κB reactivity, which is an objective value of the fraction of spontaneous basal nuclear activation over the total cytoplasmic fraction. N/C ratio are given in Table 2. It suggests that basal activation of NF-κB occurs and may participate in inhibition of apoptosis in malignant melanoma.

The reliable prognostic criteria of primary cutaneous melanoma is tumor thickness and level of invasion, and it is known that the biologic behavior of lesions correlates with thickness of the melanoma[21]. Breslow microstage is a useful index that evaluates tumor thickness in the cutaneous melanoma and Clark's classification level is useful in measuring level of invasion[21]. They are predictors of patient prognosis in cutaneous melanoma[22]. As Breslow microstage and Clark's classification level increase, the 5 year survival rate decreases. On the basis of previous results, we tried to evaluate basal nuclear activation of NF-κB as to whether it is valuable in predicting the prognosis of melanoma or not. The result showed no correlation, that is, basal activation of NF-κB may not be useful as prognostic factor of malignant melanoma. Future studies including variable prognostic factors such as survival rate, metastasis, duration, and the nuclear localization of other dimeric complexes of NF-κB such as p52/p65, p50/p50, p52/p52, RelA/RelA, RelA/c-Rel may be necessary to reveal more precise and consistent findings on the relationship with melanoma and NF-κB expression.

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