The expression of NAD(P)H:quinone oxidoreductase 1 is increased along with NF-κB p105/p50 in human cutaneous melanomas

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Abstract. NAD(P)H:quinone oxidoreductase 1 (NQO1) is a key enzyme involved in metabolism of quinones and may perform multiple functions within the cell. Recent studies demonstrated that NQO1 is overexpressed in many types of tumors, including the lung, ovary, adrenal gland, thyroid, liver, colon, breast, and pancreas. To investigate whether NQO1 plays a role in melanoma pathogenesis, we used tissue microarray technology and immunohistochemistry to examine NQO1 expression in 56 dysplastic nevi and 93 primary melanoma biopsies. Our data showed that NQO1 expression is significantly increased in primary melanomas compared with dysplastic nevi (P=0.015, χ^2 test). Our results also revealed that the increase of NQO1 was not associated with patient age, tumor thickness, ulceration, tumor site, American Joint Committee on Cancer (AJCC) stage, and 5-year patient survival. Interestingly, we found that female patients had more NQO1 expression than male patients (P=0.022, χ^2 test). Furthermore, NQO1 expression level was significantly higher in superficial spreading melanomas compared with other tumor subtypes (P=0.020, χ^2 test). Moreover, we found that NQO1 expression is significantly correlated with the expression of NF-κB subunit p50 (P=0.032, χ^2 test). Our findings suggest that NQO1 may play an important role in the initiation stage of melanoma development.

Introduction

Cutaneous malignant melanoma is a type of skin cancer originating from neoplastic proliferation of melanocytes in the epidermis. Due to its rapid lymphogenic and haematogenic

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metastasis, melanoma is one of the most malignant human tumors (1). Melanoma comprises only 4-5% of all skin cancers, but it is responsible for ~80% of deaths from skin cancer (2). Data from the National Cancer Institute (NCI) estimate that 62,480 new cases of melanoma of the skin are diagnosed in the United States this year, and ~8,420 of patients will die from this disease (3). Although melanomas at early stages are curable with surgical excision, up to 20% of patients will develop metastatic tumors owing to its high capability of invasion and rapid metastasis to other organs (4,5). Patients with metastatic melanoma have a poor prognosis, with a median survival of only 6-10 months and <5% of patients surviving more than 5 years (5-8). Therefore, understanding of the molecular events and mechanisms underlying the tumor initiation and progression, which could contribute to early detection, will be helpful for prevention and treatment of melanoma.

NAD(P)H:quinine oxidoreductase-1 (NQO1) is primarily a cytosolic enzyme that uses NADH or NADPH to directly reduce quinones to hydroquinones (9). The enzyme is generally considered as a detoxification enzyme because of its ability to detoxify reactive quinones and quinine-imines to less reactive and less toxic hydroquinones (10). This two-electron reductase also bypasses the highly reactive semi-quinone radical intermediate and prevents redox cycling (9). Through the detoxification of quinones, NQO1 prevents the formation of reactive oxygen species (ROS) and plays an important role in the cellular antioxidant system (11-14). However, the conclusions on the biological functions of NQO1 in cancer were contradictory. On one hand, NQO1 catalyzes the bio-activation of cytotoxic quinones, thereby, increasing their antitumor efficacy (15,16). On the other hand, reductive activation of environmental carcinogens including dinitro-pyrenes and heterocyclic amines by NQO1 could contribute to carcinogenesis (17). Interestingly, NQO1 was found to be expressed at high levels in many solid tumors, such as adrenal gland, breast, colon, lung, ovary, pancreas and thyroid (18-22).

To date, studies on the regulation of NQO1 gene expression have shown a complex molecular pathway. The involvement of the nuclear factor kappaB (NF-κB) signalling pathway in the induction of NQO1 gene expression has been demon-strated in different animal models and cell lines (23,24). Several studies showed that induction of *NQO1* gene by

hypoxia and mitomycin C in human colon adenocarcinoma HT29 and human hepatoma HepG2 cells is mediated through a mechanism involving the NF-κB signalling pathway (23,24). NF-κB has been shown to play an important role in melanoma proliferation, resistance to apoptosis, invasion, and metastasis (25). Our previous studies has demonstrated that NF-κB p105/p50 nuclear staining is increased in melanoma compared with dysplastic nevi (26).

Despite the extensive interest in NQO1, there is little information regarding its expression in melanoma. The aim of the present study was to determine the expression of NQO1 in different stages of human melanocytic lesions and to further investigate the role of NQO1 activity in melanoma progression using tissue microarray (TMA) technology and immunohistochemistry. Our data showed that NQO1 expression is significantly increased in primary melanomas compared to dysplastic nevi. However, increased NQO1 expression is not correlated with 5-year patient survival. We also examined the correlation of NQO1 and NF-κB p105/p50 nuclear expression in 93 cases of primary melanoma. Our data showed that NF-κB nuclear staining is significantly increased with NQO1 expression, which indicates that NQO1 expression may be regulated through NF-κB signalling pathway.

Materials and methods

TMA construction. Formalin-fixed, paraffin-embedded tissue blocks containing 66 dysplastic nevi, 118 primary melanomas were used in our present study. All specimens were obtained from the 1990-1998 archives of the Department of Pathology at Vancouver General Hospital. The use of human skin tissues in this study was approved by the medical ethics committee of the University of British Columbia and was performed in accordance with the Declaration of Helsinki guidelines. For each case, the most representative tumor area was carefully selected and marked on the hematoxylin and eosin (H&E)-stained slide. The TMAs were assembled using a tissue-array instrument (Beecher Instruments, Silver Spring, MD). Taking into account the limitation of the representative areas of the tumor, we used duplicate 0.6 mm-thick tissue cores from each donor block. Multiple 4 μm-sections were cut with a Leica microtome (Leica Microsystems Inc., Bannockburn, IL) and then transferred to adhesive-coated slides using routine histology procedures. One section from each TMA was routinely stained with H&E. The remaining sections were stored at room temperature for immunohistochemical staining.

Immunohistochemistry of TMA. The TMA slides were heated at 55˚C for 30 min to be dewaxed, and then washed with xylene 3 times, 5 min each. The tissues were then rehydrated in a series of 5-min washes in 100, 95, 80% ethanol and distilled water. Antigen retrieval was performed by heating the samples at 95˚C for 30 min in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min, and nonspecific binding was blocked by universal blocking serum (Dako Diagnostics, Mississauga, ON, Canada) for 30 min. The primary mono-clonal mouse anti-NQO1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was diluted 1:100 and incubated at 4˚C
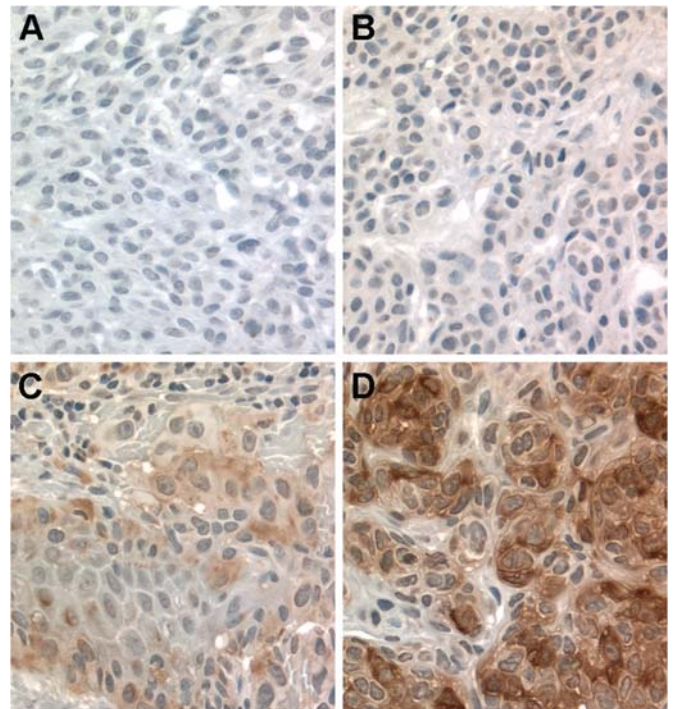

Figure 1. Representative images of NQO1 immunohistochemical staining in human melanocytic lesions. Dysplastic nevus with negative (A) or weak NQO1 expression (B). Primary melanoma with moderate (C) or strong NQO1 expression (D). Magnification, x400.
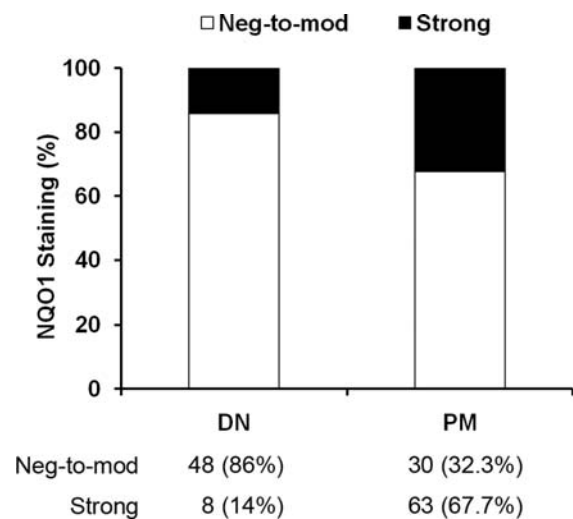

Figure 2. NQO1 expression in dysplastic nevi (DN) and primary melanoma (PM). Negative-to-moderate staining (0-9); strong staining (12). A significantly higher expression of NQO1 was found in primary melanoma when compared to dysplastic nevi (P=0.015, χ^2 test).

overnight. After 3 washes, 2 min each with PBS, the sections were incubated with biotin-labeled secondary antibody and streptavidin-peroxidase for 30 min each (Dako Diagnostics). The samples were then developed with 3,3'-diaminobenzidine substrate (Vector Laboratories, Burlington, Ontario, Canada) and counterstained with hematoxylin. Then the slides were dehydrated following a standard procedure and sealed with
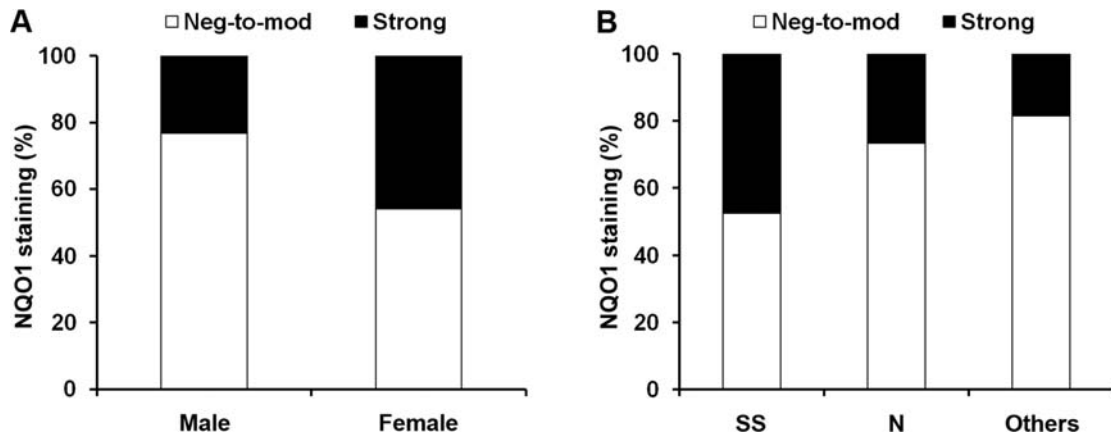
Figure 3. Correlation between NQO1 expression and clinicopathological parameters. (A) Female patients showed a significantly higher percentage of strong NQO1 expression than male patients (P=0.022, χ^2 test). (B) NQO1 expression is significantly higher in superficial spreading melanomas than other subtypes (P=0.020, χ^2 test).

coverslips. Negative controls were performed by omitting NQO1 antibody during the primary antibody incubation.

Evaluation of immunostaining. The staining intensity and percentage of NQO1 positive cells were evaluated in a blinded manner by three independent observers (including one dermatopathologist) simultaneously, and a consensus score was reached for each core. Due to loss of biopsy cores, 56 cases of dysplastic nevi and 93 cases of primary melanoma could be evaluated for NQO1 staining. NQO1 staining intensity was scored 0-3 (0 negative; 1 weak; 2 moderate; 3 strong). The percentage of NQO1 positive cells was scored into 4 categories: 1 (0-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). In the cases with a discrepancy between duplicated cores, the higher score from the two tissue cores was taken as the final score. The multiplication of the intensity and percentage scores is used as the final staining score. The final score for NQO1 staining was defined as: negative (0), weak (1-4), moderate (6-9) and strong (12).

Statistical analysis of TMA. The SPSS version 11.5 software (SPSS, Chicago, IL) was used for the statistical analysis and all tests of statistical significance were two-sided. We used the χ^2 test to compare the NQO1 staining intensity in different melanocytic lesions, as well as the correlation between NQO1 staining and the clinicopathological parameters of the melanoma patients, including age, gender, tumor thickness, ulceration, histological subtype and tumor location. The Kaplan-Meier survival curve and log-rank test were used to evaluate the correlations between NQO1 expression and patient survival. P<0.05 was considered statistically significant.

Results

Clinicopathological features of TMAs. The clinicopathological features of the melanomas for this study are summarised in Table I. Ninety-three cases of primary melanoma (56 male and 37 female) were available for the evaluation of NQO1 staining. The median age of the patients was 57 years ranging from age 21 to 93 years. For melanoma staging, we used

Breslow thickness and AJCC stages as our criteria for NQO1 expression. In our study, 30 melanoma cases were ≤1 mm, 31 were 1.01-2.0 mm, 14 were 2.01-4.0 mm, 18 were >4 mm thick; 48 tumors were at AJCC stage I, 41 were at AJCC stage II, and four were at stage III. For the tumor subtype, there were 40 cases of superficial spreading melanoma, 15 cases of nodular melanoma, 38 cases of other subtypes, including lentigo mligna melanoma, acrolentigous melanoma, and desmoplastic melanoma. The majority of the melanomas (75 cases) were located in sun-protected sites (trunk, arm, leg and feet), while 18 cases located in sun-exposed sites (head and neck). Tumor ulceration was found in 13 patients.

NQO1 expression is increased in primary melanomas. We examined overall NQO1 staining in dysplastic nevi and primary melanomas by immunohistochemistry (Fig. 1). In primary melanoma, strong NQO1 staining was observed in 30 cases (32.3%) while 63 cases (67.7%) had negative-to-moderate NQO1 staining. In dysplastic nevi, 48 cases (87%) had negative-to-moderate NQO1 expression, while only 8 cases (14.3%) showed strong staining (Fig. 2). A significant difference in NQO1 staining was observed between dysplastic nevi and primary melanoma (P=0.015, χ^2 test).

Correlation between NQO1 expression and clinicopathological parameters or 5-year patient survival. To assess whether NQO1 expression correlates with clinicopathologicial parameters of the patients, we examined the expression of NQO1 in 93 primary melanomas at various stages of invasion. We analyzed the expression level of NQO1 in tumors with different thickness and AJCC stages, as tumor thickness and AJCC stages are well known prognostic markers for patients with primary melanomas. We did not find a significant correlation between NQO1 expression and tumor thickness or AJCC stages (Table I). Interestingly, strong NQO1 expression was detected in 54% of female patients compared to only 25% of male patients who had strong NQO1 expression (P=0.022, χ^2 test; Fig. 3A). In addition, strong NQO1 expression was observed in 48% of superficial spreading melanomas compared with 27% of nodular melanomas and

Table I. NQO1 expression and clinicopathological characteristics of 93 primary melanomas.

| | NQO1 staining | | | | | |
	Negative	Weak	Moderate	Strong	Total	P-value[a]
Age						
≤57	9 (19%)	10 (22.3)	8 (17%)	20 (43%)	47	
>57	8 (17%)	16 (35%)	12 (26%)	10 (22%)	46	P>0.05
Gender						
Male	14 (25%)	17 (18%)	12 (32%)	13 (25%)	56	
Female	3 (8%)	9 (22%)	8 (16%)	17 (54%)	37	P<0.05
Tumor thickness (mm)						
≤1	1 (3%)	8 (27%)	6 (20%)	15 (50%)	30	
1.01-2	5 (16%)	10 (32%)	8 (26%)	8 (26%)	31	
2.01-4	3 (21%)	4 (29%)	3 (21%)	4 (29%)	14	
>4	8 (44%)	4 (22%)	3 (17%)	3 (17%)	18	P>0.05[b]
Ulceration						
Absent	13 (16%)	24 (30%)	20 (25%)	23 (29%)	80	
Present	4 (31%)	2 (15%)	0 (0%)	7 (54%)	13	P>0.05
Tumor subtype[c]						
SSM	4 (10%)	7 (18%)	10 (25%)	19 (48%)	40	
NM	4 (27%)	3 (20%)	4 (27%)	4 (27%)	15	
Other	7 (18%)	17 (45%)	7 (18%)	7 (18%)	38	P<0.05
Site[d]						
Sun-exposed	4 (22%)	6 (33%)	3 (17%)	5 (28%)	18	
Sun-protected	13 (17%)	20 (27%)	17 (23%)	25 (33%)	75	P>0.05
AJCC						
I	4 (8%)	13 (27%)	11 (23%)	20 (42%)	48	
II	12 (29%)	12 (29%)	7 (17%)	10 (24%)	41	
III	1 (25%)	1 (25%)	1 (25%)	1 (25%)	4	P>0.05

[a]χ^2-test for negative-to-moderate versus strong NQO1 expression. [b]Tumors, ≤2 mm versus >2 mm. [c]SSM, superficial spreading melanoma; NM, nodular melanoma; other includes desmoplastic melanoma, acrolentigous melanoma and lentigomeligna melanoma. [d]Sun-protected sites: trunk, arm, leg and feet; sun-exposed sites: head and neck.

18% of other tumor subtypes (P=0.020, χ^2 test; Fig. 3B). No correlation was found between NQO1 expression and patient age, tumor site, or ulceration status of tumors (Table I). We then examined if NQO1 expression was associated with the survival of patients with primary melanoma by Kaplan-Meier survival analysis. Our results showed that NQO1 expression did not correlated with 5-year overall and disease-specific (Fig. 4) patient survival in primary melanoma (P>0.05, long-rank test).

Correlation between NQO1 and NF-κB subunit p50 expression. Since our previous studies demonstrated that NF-κB p105/p50 nuclear staining increased with melanoma progression, and strong NF-κB p105/p50 nuclear staining was inversely correlated with disease-specific 5-year survival of patients with tumor thickness >2 mm (26), we examined the correlation of NQO1 and NF-κB p105/p50 nuclear expression in 92 cases of primary melanoma. As shown in Fig. 5, 46 cases (61%) of

strong NQO1 staining cases showed moderate to strong NF-κB staining; while only 5 cases (31%) of negative to moderate NQO1 staining cases showed moderate to strong NF-κB staining (P=0.032, χ^2 test). Our data demonstrated that NF-κB nuclear staining is significantly increased with NQO1 expression (Fig. 5).

Discussion

An important phenomenon observed in a variety of human tumors at several sites is the increased expression of NQO1, suggesting that elevated levels of this enzyme may provide an effective biomarker for neoplasia (27). However, study on the role of NQO1 in melanoma is limited. The purpose of this study was to gain information on the role of NQO1 in melanoma progression. We used TMA technology and immunohistochemistry to investigate NQO1 expression in 93 primary melanoma and 56 dysplstic nevi biopsies. Results
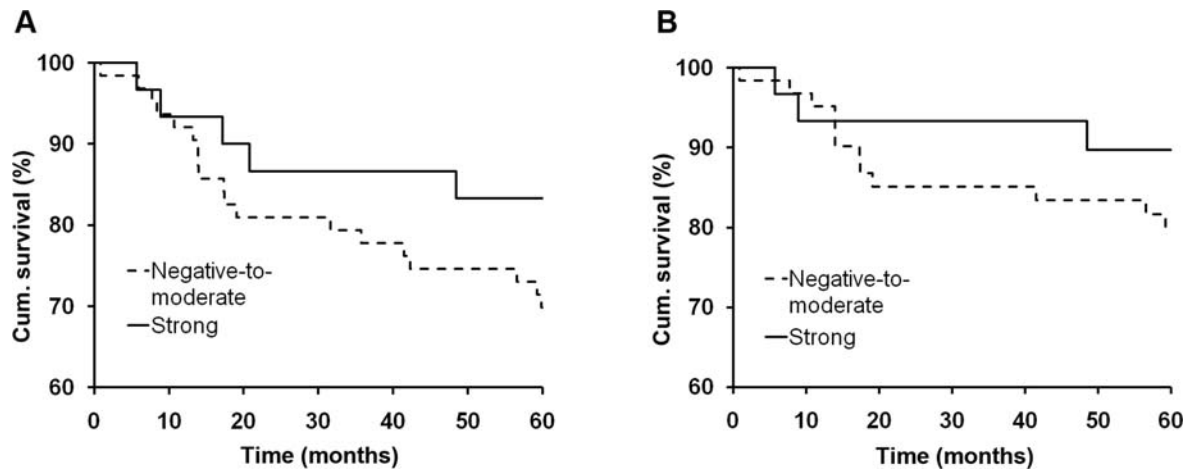
Figure 4. Correlation between NQO1 expression and 5-year survival of primary melanoma patients. There is no significant correlation of NQO1 expression with overall 5-year survival (A) and disease-specific 5-year survival (B) (P>0.05, long-rank test).
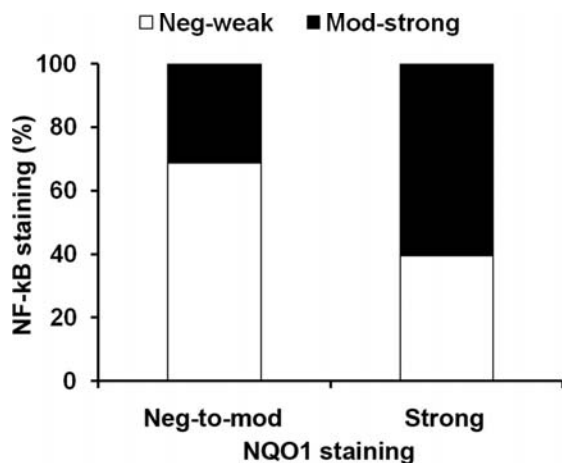

Figure 5. Correlation between NQO1 and NF-κB expression in 92 cases of primary melanoma. NF-κB expression was positively associated with NQO1 expression (P=0.032, χ^2 test).

from current study demonstrate that higher levels of NQO1 expression occur in primary melanoma tissue as has been reported for malignant tissue of the colon, breast, lung, pancreas, and liver when compared to normal tissue (18,20,28,29). Our data imply the critical role of NQO1 in the initiation of melanomagenesis. NQO1 has long been viewed as a 'good' protein that protects humans from geno-toxic damage. Additionally, it has been found that NQO1 is required in the stabilization of tumor suppressor p53, which supports the protective role of NQO1 against cancer (30). However, there is mounting evidence supporting a positive role for NQO1 in cancer progression. NQO1 is overexpressed in various tumors as mentioned above.

In another study, protein expression and enzyme activity of NQO1 was increased in colon and gastric carcinoma cell lines and in colorectal tumor samples compared to peripheral normal samples (22). These data indicate that NQO1 has potential dual roles in cancer. One possible explanation for high levels of NQO1 in tumors is that NQO1 protects not only normal cells from transforming into cancer cells, but also promotes the survival of cancer cells under a deleterious

environment. Recent study in our laboratory demonstrates that NQO1 expression significantly induces cell cycle progression by upregulating the expression of cyclin A2, B1 and D1, leading to the proliferation of melanoma cells, which may account for the overexpression of NQO1 in primary melanoma (unpublished data). In addition, NQO1 overexpression is thought to be accompanied by an increase in other anti-oxidant enzymes, such as HMOX-1 and GST, providing tumors with increased protection against cytotoxic agents allowing for rapid cancer progression (31). However, we did not find any significant correlation of NQO1 expression with tumor thickness, AJCC stages (Table I), or 5-year overall and disease-specific patient survival in primary melanomas (Fig. 4).

Interestingly, there was a higher percentage of strong NQO1 expression in female patients than male patients (Fig. 3A). This finding is consistent with the result of a study conducted on rats. In Sprague Dawley rats, gender dimorphic expression of NQO1 was observed with female mRNA, protein, and activity levels being significantly higher than in males (32). As shown by many studies, male patients may have thicker tumors at diagnosis than female patients (33-35). However, in our study, when we compared the number of tumors ≤2.0 mm or >2.0 mm, there was no difference between male and female groups (P=0.75, χ^2 test). We also found higher expression of NQO1 in superficial spreading melanomas than nodular or other types melanomas (P=0.020, χ^2 test). A recent microarray study of primary and metastatic melanoma indicated that superficial spreading and nodular melanoma had different gene expression patterns (36). Different NQO1 expression level is probably due to the genetic difference among the subtypes. The mechanisms of higher NQO1 expression in female patients and superficial spreading melanomas, and their clinical significance remain to be determined.

The factors underlying the increased levels of NQO1 in primary melanoma tissue need to be established. Expression of NQO1 is known to be induced in response to a variety of xenobiotics including polycyclic aromatic hydrocarbons, planar aromatic compounds, antioxidants, oxidants, and tumor promoters (37). As a key enzyme involved in defense against

reactive forms of oxygen, expression of NQO1 is thought to be transcriptionally controlled. Several cis-elements have been identified in the NQO1 gene promoter, including an antioxidant response element (ARE), a basal element, and AP2 element. Nuclear transcription factors Nrf2 and c-Jun bind to the ARE and activate gene expression. A NF-κB response element may also be involved in NQO1 induction (24).

Recent studies suggested that NF-κB is the major transcription factor that has been shown to play a critical role in the modulation of *NQO1*. Several studies also showed that induction of *NQO1* gene by hypoxia and mitomycin C in human colon adenocarcinoma HT29 and human hepatoma HepG2 cells is mediated through a mechanism involving the NF-κB signaling pathway (23,24). NF-κB has been shown to play an important role in melanoma cell proliferation, resistance to apoptosis, invasion, and metastasis (25). In addition, our previous studies have demonstrated that NF-κB p105/p50 nuclear staining increased with melanoma progression (26). Based on these findings, we investigated the correlation between NQO1 and NF-κB expression. Using the same tissue microarray and same protocol for immunohistochemistry staining, our analysis revealed a significant correlation between NQO1 and NF-κB expression (Fig. 5). The data indicated that NF-κB signaling pathway may be involved in the induction of *NQO1* gene expression. NF-κB is a pivotal transcription factor involved in a wide range of physiological functions, such as B-cell proliferation, cell cycle control, apoptosis, and carcinogenesis (38,39). Many stimuli activate NF-κB, including cytokines, activators of protein kinase C, viruses, and oxidants (40,41). Upon activation, NF-κB regulates the expression of many genes involved in cell proliferation, tumor invasion, immune and inflammatory responses (41). NQO1 is probably one of NF-κB regulated genes involved in cell proliferation but not in tumor invasion.

Collectively, our data indicate that NQO1 expression is significantly increased in primary melanoma compared to dysplastic nevi, but not correlated to tumor thickness or patient survival, suggesting that NQO1 may play an important role in melanoma initiation.

Acknowledgements

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