Hanyang Lin,¹ Ronald P.C. Wong,¹ Magdalena Martinka,² and Gang Li¹

Loss of SNF5 Expression Correlates with Poor Patient Survival in Melanoma

Abstract

Purpose: Aberrant expression of SWI/SNF chromatin remodeling complex is involved in cancer development. The tumor suppressor SNF5, the core subunit of SWI/SNF complex, has been shown to regulate cell differentiation, cell cycle control, and apoptosis. To investigate the role of SNF5 in the development of melanoma, we examined the expression of SNF5 in melanocytic lesions at different stages and analyzed the correlation between SNF5 expression and clinicopathologic variables and patient survival.

Experimental Design: Using tissue microarray and immunohistochemistry, we evaluated SNF5 staining in 51 dysplastic nevi, 88 primary melanomas, and 48 metastatic melanomas. We studied chemosensitivity of melanoma cells with reduced SNF5 expression by siRNA using cell survival and apoptosis assays.

Results: SNF5 expression was reduced in metastatic melanoma compared with dysplastic nevi (P = 0.005), in advanced primary melanoma (Clark's level V) compared with low risk Clark's level II melanoma (P = 0.019), and in melanoma at sun-exposed sites compared with sun-protected sites (P = 0.044). Furthermore, we showed a strong correlation between negative SNF5 expression and a worse 5-year survival in melanoma patients (P = 0.016). Multivariate Cox regression analysis revealed that negative SNF5 expression is an independent prognostic factor to predict patient outcome in primary melanomas (P = 0.031). Finally, we showed that knockdown of SNF5 in melanoma cell lines resulted in significant chemoresistance.

Conclusions: Our data indicate that SNF5 may be an important marker for human melanoma progression and prognosis as well as a potential therapeutic target. (Clin Cancer Res 2009;15(20):OF1–8)

Human cutaneous malignant melanoma is a life-threatening skin cancer, for its ability to metastasize rapidly and its resistance to conventional radiotherapy and chemotherapy (1, 2). Although melanoma accounts for only 4% of skin cancers, it is responsible for 80% of deaths from skin cancer (3). Furthermore, although melanoma is curable through early diagnosis and surgical excision (4), up to 20% of patients will develop metastatic tumor due to its highly invasive and metastatic properties (5). Consequently, metastatic melanoma patients have a poor prognosis, with median survival of only 6 to 10 months and <5% of the patients surviving >5 years (4, 6, 7).

SWI/SNF ATP–dependent chromatin-remodeling complex is a 2-Mda multisubunit complex first identified in yeast and highly conserved among eukaryotes (8). SWI/SNF complexes play essential roles in transcriptional regulation, contribute to the control of cellular processes, such as proliferation and differentiation, and also are involved in DNA repair by altering the accessibility of UV-damaged DNA-binding proteins to DNA lesions (9–13). SNF5, also known as INI1/BAF47/SMARCB1, is the core subunit of mammalian SWI/SNF complex. Many genetic evidences have defined SNF5 as a tumor suppressor gene in humans and mice. In mice, homozygous deletion of SNF5 is embryonic lethal, and heterozygous mice are predisposed to develop tumors due to loss of heterozygosity for SNF5 (14–16). In humans, homozygous inactivating mutations or deletions of the SNF5 gene are associated with malignant rhabdoid tumors (MRTs) (17–19).

Studies have shown that SNF5 exhibits an antiproliferative activity in MRTs because SNF5 overexpression leads to a G1 cell cycle arrest associated with an increase in p16INK4a, E2F, and Cyclin D (20–22). SNF5 is also required for neural and adipocyte differentiation in vitro (23, 24). This suggests that SNF5 is able to regulate the balance between cell proliferation and differentiation (24). Finally, the invasive property of malignant rhabdoid tumor is dramatically reduced upon SNF5 expression.

Authors’ Affiliations: Departments of ¹Dermatology and Skin Science and ²Pathology, Vancouver Coastal Health Research Institute, University of British Columbia, Vancouver, British Columbia, Canada

Received 5/4/09; revised 6/15/09; accepted 6/16/09; published OnlineFirst 10/8/09.

Grant support: Canadian Institutes of Health Research (MOP-84559 and MOP-93810) and Canadian Dermatology Foundation (G. Li), Natural Sciences and Engineering Research Council Postgraduate Scholarship (H. Lin), and National Cancer Institute of Canada PhD Studentship (R.P.C. Wong).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Gang Li, Jack Bell Research Centre, 2660 Oak Street, Vancouver, BC V6H 3Z6, Canada. Phone: 1-604-875-5826; Fax: 1-604-875-4497; E-mail: gangli@interchange.ubc.ca.

© 2009 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-09-1135

Imaging, Diagnosis, Prognosis
Translational Relevance

Our study reveals that expression level of the tumor suppressor SNF5, the core subunit of SWI/SNF chromatin remodeling complex, is reduced in metastatic melanoma, indicating an important role of SNF5 in melanoma progression. We also show that negative SNF5 expression correlates with a worse 5-year melanoma patient survival compared with those of positive SNF5 expression. Consistent with this finding, we showed that melanoma cells depleted with SNF5 expression by RNA interference render cells resistant to chemotherapeutic drugs. The highly metastatic potential and resistance to conventional radiotherapy and chemotherapy are the major reasons for which melanoma patients succumb to the disease. Therefore, strategies to restore reduced SNF5 expression may be a potential approach for melanoma therapy. Moreover, analyzing SNF5 expression in melanoma patients may predict the response to chemotherapy.

Materials and Methods

Construction of TMA. Formalin-fixed, paraffin-embedded tissues from 51 dysplastic nevi, 88 primary melanomas, and 48 metastatic melanomas were used for this study. Five cases of normal skin tissues and five cases of normal nevi were included as positive controls. All specimens were obtained from the 1990 to 1998 archives of the Department of Pathology, Vancouver General Hospital. The use of human skin tissues was approved by the medical ethical committee of the University of British Columbia and was done in accordance with the Declaration of Helsinki guidelines. The most representative tumor area was carefully selected and marked on the H&E-stained slide. The TMAs were assembled using a tissue-array instrument (Becherer Instruments). Duplicate 0.6-mm-thick tissue cores were taken from each biopsy specimen. Two composite high-density TMA blocks containing 126 and 111 cases from a total of 237 patients were designed. Using a Leica microtome (Leica Microsystems, Inc.), multiple 4-µm sections were cut and transferred to adhesive-coated slides using regular histology procedures. H&E were used to stain one section from each TMA. Other sections were kept at room temperature for immunohistochemical staining.

Immunohistochemistry of TMA. TMA slides were de waxed at 55°C for 20 min followed by three 5-min washes with xylene. The rehydration of tissues was done by 5-min washes in 100%, 95%, and 80% ethanol and distilled water. Antigen retrieval was done by heating the samples at 95°C for 30 min in 10 mmol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 30 min. After 30 min of blocking with the universal blocking serum (Dako Diagnostics), the sections were incubated with monoclonal mouse anti-SNF5 antibody (1:200 dilution; Abcam) at 4°C overnight. The sections were then incubated for 30 min each with a biotin-labeled secondary antibody and then streptavidin-peroxidase (Dako Diagnostics). The samples were developed using 3,3′-diaminobenzidine substrate (Vector Laboratories) and counterstained with hematoxylin. Dehydration was then done following a standard procedure and the slides were sealed with coverslips. Negative controls were done by omitting the SNF5 antibody during the primary antibody incubation.

Evaluation of immunostaining. The evaluation of SNF5 staining was examined by one dermatopathologist and two other observers simultaneously, and a consensus was reached for each core. Microarray

Fig. 1. Correlation between SNF5 expression and melanoma progression. A to D, representative images of SNF5 immunohistochemical staining. A, positive staining in normal skin (arrows, melanocytes); B, positive staining in benign nevus; C, positive SNF5 staining in dysplastic nevus; and D, negative SNF5 staining in primary melanoma. Magnification, ×400. E, reduced SNF5 expression correlates with melanoma progression. A significant difference of SNF5 staining was observed between dysplastic nevi (DN) and metastatic melanoma (MM; P = 0.005, χ² test).
positivity for SNF5 was defined as any detectable nuclear or cytoplasmic staining (35). Examples for positive and negative SNF5 staining are given in Fig. 1C and D.

**Cell culture and transfection.** MMRII and MEWO human melanoma cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen). All cells were maintained in 5% CO2 atmosphere at 37°C. Cells were grown to 50% confluence before small interfering RNA (siRNA) transfection. Nonspecific control siRNA or SNF5 siRNA (Qiagen) was transfected by siLentFect Lipid Reagent (Bio-Rad) according to the manufacturer's instructions. Twenty-four hours after transfection, the medium containing transfection reagents was removed. The cells were rinsed twice with PBS and incubated in fresh medium. Forty-eight hours after transfection, cells were lysed for Western blot assay, and subjected to sulforhodamine B (SRB) cell survival assay and apoptosis analysis.

**Western blot analysis.** Cells were harvested and washed thrice with PBS. Whole cell proteins were extracted as described previously (36). Protein concentrations were determined by protein assay (Bio-Rad). Western blot analysis was done as described previously (37). The following antibodies were used for Western blot: mouse anti-SNF5 (Abcam) and mouse anti-β-actin (1:1,000; Sigma). IRIRDye-labeled secondary antibody was applied to the blot for 1 h at room temperature. The signals were detected with Odyssey IR Imaging system (LI-COR).

**SRB cell survival assay.** Cells were transfected with SNF5 siRNA for 48 h, and then treated with various doses of doxorubicin, etoposide, and camptothecin (Sigma). Twenty-four hours after drug treatments, cells were fixed with 40 μg/mL doxorubicin, 5 μmol/L etoposide, and 25 mmol/L camptothecin. Twenty-four hours after drug treatments, cells were fixed with 0.1% Triton X-100, 0.1% sodium citrate, and 25 μg/mL RNase A for 30 min. Samples were then analyzed using a FACSCanto flow cytometer (BD Biosciences).

**Statistical analysis.** For TMA, statistical analysis was done with SPSS 11.5 software (SPSS). The χ² test was used to compare the quantitative differences of SNF5 staining in different stages of melanoma progression. The association between SNF5 staining and the clinicopathologic parameters of the primary melanoma patients, including age, gender, tumor thickness, ulceration, histologic subtype, location, and Clark's level, was also evaluated by χ² test. The Kaplan-Meier method and log-rank test were used to evaluate the correlation between SNF5 expression and patient survival. Cox regression model was used for multivariable analysis. For SRB cell survival and apoptosis assays, student's t test was used. A P value of <0.05 was considered significant.

**Results**

**Clinicopathologic features of TMA's.** Fifty-one cases of dysplastic nevi, 88 cases of primary melanomas, and 48 cases of metastatic melanomas are evaluated for SNF5 staining. As shown in Table 1, for the 88 cases of primary melanomas, there were 44 males and 44 females, with age ranging from 21 to 93 years (median, 57 years). For primary melanoma staging, Breslow thickness and Clark's level were used as criteria for evaluating SNF5 expression: 26 tumors were ≤1.0 mm, 30 tumors were 1.01 to 2.0 mm, 14 tumors were 2.01 to 4.0 mm, and 18 were >4.0 mm; 20 tumors were at Clark's level II, 25 tumors were at level III, 31 tumors were at level IV, and 12 tumors were at level V. Ulceration was observed in 18 cases. For the histologic subtype, 13 tumors were nodular melanomas, 39 tumors were superficial spreading melanomas, 15 tumors were lentigo maligna melanomas, and 21 tumors were nonspecified. Seventeen melanomas were located in sun-exposed sites (head and neck), and 71 were located in sun-protected sites (other locations).

**Reduced SNF5 expression correlates with melanoma progression.** To investigate if SNF5 expression is changed in pigmented melanocytic lesions, immunohistochemistry staining of dysplastic nevi, primary melanoma, and metastatic melanoma were done using TMA technique (Fig. 1). Positive SNF5 staining was recorded in 98%, 90%, and 81% of the biopsies in dysplastic nevi, primary melanoma, and metastatic melanoma, respectively (Fig. 1E). Melanocytes in all five cases of normal skin tissues and five benign nevi showed strong positive SNF5 staining (Fig. 1A). However, there is no significant difference in SNF5 stain between dysplastic nevi and primary melanoma (P = 0.069, χ² test), or between primary melanoma and metastatic melanoma (P = 0.161, χ² test).

**Reduced SNF5 expression correlates with Clark's level and tumor location.** Because Clark's level of invasion is an important prognostic marker for patients with primary melanoma, we first studied if SNF5 expression correlates with Clark's level. We found a significant difference in SNF5 expression between different stages of melanocytic lesions have positive staining, negative SNF5 staining was significantly increased in metastatic melanoma when compared with dysplastic nevi (P = 0.005, χ² test). However, there is no significant difference in SNF5 staining between dysplastic nevi and primary melanoma (P = 0.069, χ² test), or between primary melanoma and metastatic melanoma (P = 0.161, χ² test).
Clark's level V and Clark's level II \((P = 0.019, \chi^2\) test; Fig. 2A). All the tumors in Clark's level II had positive SNF5 staining, whereas only 75% of tumors in Clark's level V had positive SNF5 expression.

As UV radiation is the main environmental factor for melanoma formation, we analyzed the SNF5 staining in sun-exposed or sun-protected sites. Reduced positive SNF5 staining significantly correlated with the location of primary melanomas \((P = 0.044, \chi^2\) test; Fig. 2B). Although 93% of tumors from sun-protected sites (trunk, arm, leg, and feet) had positive SNF5 staining, SNF5 staining was reduced to 76% in tumors from sun-exposed sites (head and neck). We did not find significant correlations between SNF5 expression with other clinicopathologic variables, including Breslow tumor thickness, subtype, ulceration, or patient's age or gender (Table 1).

**Reduced SNF5 expression correlates with poor patient survival.** To evaluate whether reduced SNF5 staining in human primary and metastatic melanomas correlate with a worse prognosis, Kaplan-Meier survival curves were constructed using overall or disease-specific 5-year survival to evaluate the biopsies with positive SNF5 staining to those with negative SNF5 staining. Our data revealed that positive SNF5 staining correlated with both overall and disease-specific 5-year survival in primary melanomas \((P = 0.016\) and \(P = 0.049\), respectively, log-rank test; Fig. 3A and B). The correlation is also significant when primary and metastatic melanoma cases are combined for the analysis; both overall and disease-specific 5-year survival rates are significantly better for patients with positive SNF5 expression in their tumor tissues compared with those with negative SNF staining \((P = 0.029\) and \(P = 0.040\), respectively, log-rank test; Fig. 3C and D).

In addition, we examined whether positive SNF5 expression is an independent prognostic marker for melanoma. We performed a multivariate analysis including SNF5 expression, age, gender, tumor thickness, ulceration, location, and subtype for 88 primary melanomas. We found that similar to tumor thickness and presence of ulceration, which have been widely accepted as independent prognostic factors for melanoma patient survival, SNF5 expression is also an independent prognostic factor for both overall \([relative\ risk, 5.145; 95\%\ confidence\ interval (CI), 1.48-17.89; P = 0.010; Table 2]\) and disease-specific 5-year survival \([relative\ risk, 4.637; 95\%\ CI, 1.15-18.63; P = 0.031; Table 2]\). Our results clearly indicate that negative SNF5 expression in either primary or metastatic melanoma is associated with poor prognosis, suggesting that SNF5 reduction may serve as a molecular prognostic marker for this aggressive disease.

**Resistance to chemotherapeutic drugs increases in SNF5 knockdown melanoma cell lines.** A major obstacle in treating melanoma is its resistance to drug-induced apoptosis (39). Because negative SNF5 expression strongly correlates with poor patient survival, we investigated the involvement of SNF5 in chemo-resistance of melanoma cells. We first transiently transfected MMRU and MEWO melanoma cells with SNF5 siRNA or control siRNA. Forty-eight hours after transfection, cells were harvested for Western blot analysis (Fig. 4A) or subjected to cell survival and apoptosis assays (Fig. 4B and C). Western blot indicated that at least 75% knockdown of SNF5 protein expression in both MMRU and MEWO cells transfected with SNF5 siRNA compared with those transfected with control siRNA. In SBR cell survival assay, cell survival of both MMRU and MEWO SNF5 knockdown cells treated with doxorubicin, etoposide, and camptothecin was significant higher than those of control cells (Fig. 4B). However, we did not observe a significant difference in cell proliferation upon SNF5 knockdown in both MMRU and MEWO cells when compared with the control cells without drug treatment (Supplementary Fig. S1). To investigate if reduced chemosensitivity of SNF5 knockdown cells is due to reduced apoptosis, we performed flow cytometry analysis. Our data showed that both drug-treated MMRU and MEWO SNF5 knockdown cells have significantly lower sub-G1 populations compared with control cells (Fig. 4C). Thus, these data indicate that downregulation of SNF5 expression reduces drug-induced apoptosis in melanoma cells. This might suggest that melanoma resistance to chemotherapy is at least partially due to the reduction of SNF5 protein level.

**Discussion**

Several lines of evidence indicated that aberrant expression of the SWI/SNF chromatin remodeling complex proteins are involved in tumorigenesis. Deletion or mutation of the **BRG1** gene was found in lung, breast, prostate, and melanoma cancer cell lines (28, 30, 31, 33, 40). The **SNF5** gene was also found inactivated in MRTs and downregulated in epithelioid sarcomas.
Our result reveals a significant correlation between reduced SNF5 expression and melanoma progression (Fig. 1E). However, our finding is in contrast with the report by Hornick et al. (41) showing SNF5 expression is intact in metastatic melanoma. We argue that the discrepancy may be due to lack of proper control and the smaller sample size in their study. They tested only 20 cases of metastatic melanomas without comparison to nevi or primary melanoma biopsies. Therefore, it is inaccurate for these authors to claim that SNF5 expression is indeed intact in metastatic melanoma. Different antibodies used in the immunohistochemical studies could also be the cause of the discrepancy.

Although the difference in SNF5 expression between dysplastic nevi and primary melanoma only reaches a borderline

<table>
<thead>
<tr>
<th>Variable*</th>
<th>Overall survival</th>
<th>Disease-specific survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative risk (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>SNF5</td>
<td>5.145 (1.48-17.89)</td>
<td>0.010</td>
</tr>
<tr>
<td>Age</td>
<td>0.775 (0.28-2.12)</td>
<td>0.620</td>
</tr>
<tr>
<td>Gender</td>
<td>3.215 (1.29-8.00)</td>
<td>0.012</td>
</tr>
<tr>
<td>Thickness</td>
<td>0.296 (0.09-0.94)</td>
<td>0.039</td>
</tr>
<tr>
<td>Ulceration</td>
<td>0.293 (0.10-0.85)</td>
<td>0.024</td>
</tr>
<tr>
<td>Location</td>
<td>0.705 (0.26-1.93)</td>
<td>0.497</td>
</tr>
<tr>
<td>Subtype</td>
<td>0.476 (0.13-1.81)</td>
<td>0.276</td>
</tr>
</tbody>
</table>

*Coding of variables: SNF5 was coded as 1 (negative), and 2 (positive). Age was coded as 1 (≤57 y), and 2 (>57 y). Gender was coded as 1 (female) and 2 (male). Thickness was coded as 1 (≤2 mm), and 2 (>2 mm). Ulceration was coded as 1 (absent) and 2 (present). Location was coded as 1 (head and neck), and 2 (others). Subtype was coded as 1 (nodular) and 2 (others).
significance (Fig. 1E), which might due to insufficient sample size ($P = 0.069$, $\chi^2$ test), a trend of decreased SNF5 staining in primary melanoma was observed (2% negative SNF5 staining in dysplastic nevi versus 10% in primary melanoma). This suggests a possible role of SNF5 in the initiation of melanoma. It is also worth to note that reduced SNF5 expression correlates with tumor location at sun-exposed sites (Fig. 2B), indicating a crucial role of UV radiation in regulating SNF5 level. Reduction of SNF5 level could be due to UV-induced mutation at the SNF5 locus at 22q11.2, which is found homozygously deleted or mutated in MRTs (12). Besides the role of SNF5 in melanoma initiation, SNF5 has been shown to play a role during

**Fig. 4.** Chemotherapeutic drug-induced apoptosis is repressed in SNF5 knockdown melanoma cells. A, SNF5 protein knockdown level detected by Western blot (arrow, SNF5 band from previous blot). B, drug-induced cell survival by SRB staining. Forty-eight hours after transfection with SNF5 siRNA or control siRNA, MMRU and MEWO cells were treated with doxorubicin, etoposide, or camptothecin.Twenty-four hours after drug treatments, cells were fixed with 10% trichloroacetic acid for 1 h and quantitated by SRB staining. C, drug-induced apoptosis assayed by fluorescence-activated cell sorting. Forty-eight hours after transfection with SNF5 siRNA or control siRNA, MMRU and MEWO cells were treated with 0.25 $\mu$g/mL doxorubicin (DOX), 5 $\mu$mol/L etoposide (EP), and 25 nmol/L camptothecin (CPT) for 24 h, or without drug treatment as control (Ctrl). Cells were then stained with 40 $\mu$g/mL propidium iodide for 30 min, and the percentage of apoptotic (sub-G1) cells was measured by flow cytometry. All experiments were carried out in triplicate.
Acquired resistance to apoptosis is a hallmark of cancer (44), which allows cancer cells to escape drug-induced apoptosis and enables the establishment of metastasis. Metastatic melanoma is particularly resistant to conventional radiotherapy and chemotherapy (1, 2). A study by Oh et al. (45) indicating that doxorubicin-induced apoptosis is repressed in NIH3T3 cells transduced with dominant-negative form of BAF60a. BAF60a, a mediating subunit of SWI/SNF complex proteins, interacts with p53, and uncoupling of p53 with the SWI/SNF complex resulted in repression of apoptosis and cell cycle arrest. Because melanomas rarely harbor p53 mutations (46, 47), SNF5 might play a role in regulating apoptosis in melanoma cells. Indeed, our in vitro data revealed that knocking down SNF5 in melanoma cells with siRNA triggered stronger resistance to chemotherapeutic drugs (Fig. 4). Combined with patient survival data, this finding led us to speculate that reduced SNF5 expression may contribute to chemoresistance and thus decreased survival in melanoma patients. Nevertheless, the molecular mechanisms of SNF5 and its relations to BAF60a in apoptosis of melanoma cells after chemodrug treatment warrant further investigation.

In conclusion, the data from this study show that SNF5 expression is reduced in human cutaneous melanoma and significantly correlated with patient survival, suggesting that SNF5 plays an important role in melanomagenesis and it may serve as a promising prognostic marker and the therapeutic target for malignant melanoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Drs. D. Huntsman, N. Makretsov, and H. Masoudi for the technical assistance in TMA construction and J. Li in statistical analysis.

---

**References**


Clinical Cancer Research

Loss of SNF5 Expression Correlates with Poor Patient Survival in Melanoma

Hanyang Lin, Ronald P.C. Wong, Magdalena Martinka, et al.

Clin Cancer Res  Published OnlineFirst October 6, 2009.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-09-1135

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2009/09/15/1078-0432.CCR-09-1135.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.