Received: 30 January 2022 | Revised: 17 April 2022 | Accepted: 21 April 2022

DOI: 10.1111/cas.15379

ORIGINAL ARTICLE

Cancer Science WILEY

Association of Nrf2 expression and mutation with Weiss and Helsinki scores in adrenocortical carcinoma

Takao Kamai[1] | Satoshi Murakami[1] | Kyoko Arai[1] | Kazuyuki Ishida[2] | Toshiki Kijima[1]

[1]Department of Urology, Dokkyo Medical University, Mibu, Tochigi, Japan

[2]Department of Diagnostic Pathology, Dokkyo Medical University, Mibu, Tochigi, Japan

Correspondence
Takao Kamai, Department of Urology, Dokkyo Medical University, 880 Kitakobayashi Mibu, Tochigi 321-0293, Japan.
Email: kamait@dokkyomed.ac.jp

Funding information
This work was supported in part by the Japanese Science Progress Society KAKENHI Grants (20K09546) to T. Kamai.

Abstract

Adrenocortical carcinoma (ACC) is a rare malignant tumor. Genetic abnormalities that may represent therapeutic targets and prognostic factors in ACC remain unclear. Besides being one of the main cellular defense mechanisms that regulates antioxidant pathways for detoxifying reactive oxygen species (ROS), the transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2) promotes tumor proliferation by increasing metabolic activity. In surgical specimens from 12 cases of nonmetastatic ACCs and nine cases of benign adrenocortical adenoma (ACA), we investigated gene mutation and protein expressions for Nrf2 and the preoperative maximum standard glucose uptake (SUVmax) on [^{18}F]fluorodeoxy-glucose positron emission tomography. Three of five ACCs with a Weiss score of 7 to 9 were Nrf2 mutants; these ACCs had higher expression of Nrf2 and higher preoperative SUVmax. The other seven ACCs had a Weiss score of 3 to 6; these seven ACCs and all the ACAs were non-Nrf2 gene mutants. Patients with a Weiss score of 7 to 9 and Nrf2 mutant ACC had shorter overall survival. Based on Helsinki scoring, three ACCs with a Helsinki score greater than 17 had Nrf2 mutants, higher expression of Nrf2, higher preoperative SUVmax, and shorter overall survival. Our findings indicate that Nrf2 activation and the associated increase in metabolism play roles in ACC, in particular in ACC with a Weiss score of 7 to 9 and a Helsinki score of greater than 17.

KEYWORDS
[^{18}F]fluorodeoxy-glucose positron emission tomography (^{18}F-FDG-PET), adrenocortical carcinoma, nuclear factor E2–related factor 2 (Nrf2), p53, Weiss and Helsinki scores

1 | INTRODUCTION

In 2017, the World Health Organization (WHO) recognized adrenocortical carcinoma (ACC) as a malignant epithelial tumor of adrenal cortex cells.[1] Adrenocortical carcinoma is a highly malignant, extremely rare tumor with a frequency of about 0.5 to 2 per million people[2-7] Therefore, it is extremely important to distinguish between benign and malignant adrenocortical tumors and to accurately diagnose the extent of malignancy in appropriate treatment selection. According to the WHO, ACC should be staged with the European

Abbreviations: ^{18}F-FDG-PET, [^{18}F]fluorodeoxy-glucose positron emission tomography; ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; ARE, antioxidant response element; bZip, basic region-leucine zipper; CT, computed tomography; ENS@T, European Network for the Study of Adrenal Tumors; FIRM-ACT, the First International Randomized trial in locally advanced and Metastatic Adrenocortical Carcinoma Treatment; Keap1, Kelch-like ECH-associated protein 1; MRI, magnetic resonance imaging; Nrf2, nuclear factor erythroid 2–related factor 2; ROS, reactive oxygen species; SNP, single-nucleotide polymorphisms; SNV, single-nucleotide variants; SUVmax, maximum standard glucose uptake; WHO, World Health Organization.

Network for the Study of Adrenal Tumors (ENS@T) system, which considers tumor size and extent[2-7] Surgery is the main treatment for nonmetastatic ACC, but recurrence is frequent even after complete removal. The treatment recommendations in the revised 2018 ENS@T treatment guideline for ACC[7] are gaining increasing attention; however, effective treatment for ACC has not yet been established, and few treatment options are available. Therefore, to enable appropriate treatment selection, it is important to distinguish between benign and malignant adrenocortical tumors and to diagnose malignancy.

Histopathology is the primary determinant of malignancy. The most widely used criteria for diagnosing benign and malignant ACC were published by Weiss in 1984 and refer to morphological findings.[8] The scoring system includes nine diagnostic criteria: a tumor that fulfils three or more criteria is assumed to be malignant, and one that fulfils less than three criteria, benign. However, interobserver variance between diagnosticians often is a problem, and even experienced pathologists specialized in endocrine pathology frequently do not agree on the Weiss score. Therefore, alternative scoring systems were proposed, namely, the Weiss revisited score (in 2002) and the Helsinki score (in 2015).[9,10] Evaluations of the ability of the various scoring systems to discriminate between benign and malignant tumors by assessing the presence or absence of metastatic lesions found that all three scores have good sensitivity and specificity, as follows: Weiss score, 100% sensitivity and 90.2% specificity; Weiss revisited score, 100% and 96.9%, respectively; and Helsinki score, 100% and 99.4%, respectively.[9,10]

Because most cases of metastatic ACC are less well–differentiated tumors and have a high score in all three scoring systems, diagnosis is almost always straightforward. In contrast, distinguishing between benign adrenocortical adenoma (ACA) and localized malignant ACC (including resectable cases) is problematic; however, postoperative treatment options depend on whether the tumor is benign or malignant. The most useful index is the nuclear protein Ki-67, an immunohistochemical auxiliary diagnostic marker assessed with MIB-1 monoclonal antibodies. For example, in addition to the morphological findings, the Helsinki score incorporates the Ki-67 labeling rate. Ki-67 is useful not only for differentiating benign from malignant adrenocortical tumors, but also for determining prognosis. Although there is still no fixed method for determining the Ki-67 labeling rate, ACC has remarkable intratumoral heterogeneity, and the Ki-67 labeling rate in the tumor depends on whether Ki-67 labels a hot spot or the entire tumor.[11] Therefore, the interobserver variance for Ki-67 labeling rate may also increase depending on the measurement location.[11] Currently, no sufficiently validated index for assessing ACC is available.

In recent years, many studies investigated the association of gene mutations with the development of ACC.[3,4] Although the Wnt/β-catenin signaling pathways were confirmed as frequently altered pathways in ACC, mutations in the β-catenin gene, *CTNNB1*, were detected in approximately 40% of ACCs and benign ACAs,[12,13] indicating that the Wnt/β-catenin pathway might be not sufficient for malignant transformation.[3,4] The p53 gene mutation was detected in about one-third of pediatric and adult cases.[12,13] In addition, about 30% of cases had mutations in genes such as *TERT*, *CDK4*, *ZNRF3*, and *RB1*, and myxoid variants of ACC frequently had *CDK4* and *RB1* mutations.[12,13] However, among the effects of the abovementioned gene mutations, none appears to be a therapeutic target or prognostic factor. At present, no morphological findings are typified by both the Weiss criteria and genetic abnormalities, and whether genetic abnormalities exist that may serve as therapeutic targets and prognostic factors in ACC remains unclear. Thus, pathways potentially involved in the pathogenesis of ACC need to be identified, and studies need to elucidate genetic abnormalities that affect the progression of ACC.

Cancer cells undergo metabolic reprogramming and generate energy by aerobic glycolysis, a characteristic known as the Warburg effect.[14] They have elevated levels of protein synthesis and energy generation and meet the associated increased nutrient demand by vasculogenesis and/or by higher nutrient uptake through upregulation of glucose and monocarboxylate transporters, among others.[15] An important role in this biological response is played by the transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2) pathway. Reactive oxygen species (ROS) promote carcinogenesis by creating oxidative stress, which affects biopolymers such as DNA, proteins, and lipids. In response to this oxidative stress, cells activate antioxidant pathways that involve Nrf2. In addition to being involved in these cellular defense mechanisms, research indicates that Nrf2 promotes proliferation of tumors by increasing metabolic activity.[16,17] In normal tissues, inhibition of Nrf2 signaling prevents carcinogenesis and tumor progression, and in malignant cells, activation of Nrf2 signaling promotes chemoresistance and proliferation.[18,19] Thus, constitutive Nrf2 activation might be linked with the development and progression of human cancers. High Nrf2 expression appears to be associated with poor prognosis in ACC[20]; however, a possible association of Nrf2 with ACC has yet to be fully elucidated. Therefore, we investigated Nrf2 gene mutation by targeted next-generation sequencing and Nrf2 protein expressions by immunohistochemistry in surgical specimens from 12 patients with nonmetastatic ACCs at surgery and, as a control group, nine patients with benign ACAs who underwent surgical resection. In addition, previous studies showed that [^{18}F]fluorodeoxy-glucose positron emission tomography (^{18}F-FDG-PET) was highly sensitive and specific for differentiating malignant from benign adrenal disease, indicating that ACC might have metabolic characteristics.[21] The metabolic activity of cells in ACC has also yet to be fully studied by ^{18}F-FDG-PET, so we assessed Nrf2 gene mutation and protein expression and preoperative maximum standard glucose uptake (SUVmax) on ^{18}F-FDG-PET in ACC with the aim to increase knowledge on the role of the Nrf2 pathway in metabolism in ACC.

2 | METHODS

2.1 | Patients

This was a retrospective study of 21 patients (16 men and five women; median age, 53 years; range, 41–67 years) with histopathologically diagnosed nonmetastatic ACC (n = 12; nine men and three women)

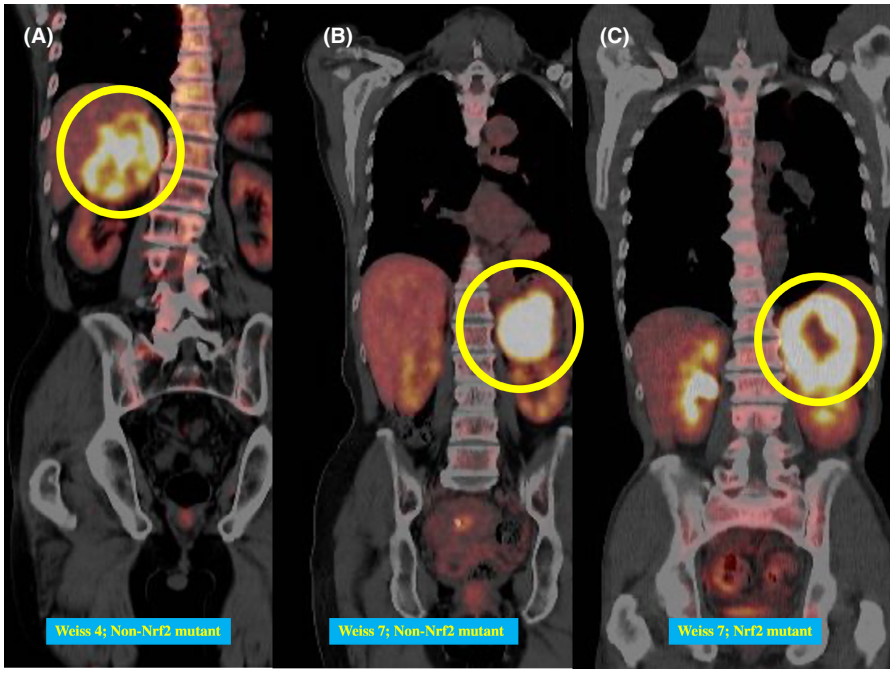

FIGURE 1 ^{18}F-FDG-PET/CT. A, 71-y.o. male patient with adrenocortical carcinoma (ACC) with a Weiss score of 4, a Helsinki score of 6.3, and without Nrf2 gene mutation. SUVmax: 8.1. B, A 66-y.o. female patient with ACC with a Weiss score of 7, a Helsinki score of 11.7, and without Nrf2 gene mutation. SUVmax: 14.7. C, A 67-y.o. male patient with Nrf2 mutant ACC with a Weiss score of 7 and a Helsinki score of 19.3. SUVmax: 19.1

or ACA (n = 9; seven men and two women) who underwent surgical resection for the tumor at Dokkyo Medical University Hospital between 2011 and 2020. Before surgery, staging was performed in all patients by computed tomography (CT) and/or magnetic resonance imaging (MRI). Furthermore, whole-body imaging was performed with a combined ^{18}F-FDG-PET/CT scanner (^{18}F-FDG-PET/CT; Biograph, Sensation 16, Siemens Systems). The baseline SUVmax was the maximum preoperative SUVmax value on ^{18}F-FDG-PET (Figure 1). Tumors were classified with the Weiss criteria,[7] so tumors that fulfilled three or more of the nine criteria were assumed to be malignant, and those that fulfilled fewer than three criteria, benign. Tumors were also classified into four groups using the modified score according to the

TABLE 1 Pathological features and gene mutation and protein expression of Nrf2

Helsinki score					Weiss criteria			
Case/Sex	A. Mitotic rate	B. Necrosis	C. Ki−67	Helsinki score	D. High nuclear grade	E. Mitotic count score	F. Atypical mitosis	G. Clear cell
	0: ≤5	0: absence	%		0: negative	0: ≤5	0: absence	0: ≥25%
	3: >5	5: presence			1: positive	1: >5	1: presence	1: <25%
				A+B+C	Mitotic rate greater than 5 per 50 high-power fields		Clear lipid-rich cells comprising less than 25% of the tumor	
>3, malignant								
1. M	3	5	16	24	1	1	0	1
2. M	3	5	65	73	1	1	1	1
3. M	3	0	30	33	1	1	0	1
4. M	0	5	6	11	0	0	0	0
5. M	0	5	18	23	1	0	0	1
6. M	3	5	56	64	1	1	0	1
7. M	3	0	30	33	1	1	0	1
8. F	3	0	30	33	1	1	0	1
9.M	3	5	9	17	1	1	0	1
10. M	3	0	8	11	1	0	0	1
11. M	3	5	45	53	1	1	0	1
12. F	3	0	7	10	0	1	0	0

Helsinki score[10]: (1) benign tumors with a Helsinki score 0 to 8.5, (2) malignant tumors with a Helsinki score 8.5 to 17, (3) malignant tumors with a Helsinki score 17 to 34, and (4) malignant tumors with a Helsinki score greater than 34.

If the ACC recurred after complete resection, patients were treated with mitotane with or without cytotoxic agents (including etoposide, doxorubicin, and cisplatin), as defined in the First International Randomized trial in locally advanced and Metastatic Adrenocortical Carcinoma Treatment (FIRM-ACT).[1,5,22]

2.2 | DNA extraction

After freezing in liquid nitrogen, tumor samples were ground to a powder. DNA was extracted from the powder (30–50 mg) with an AllPrep kit (Qiagen). The amount and purity of the DNA in the samples were measured with a NanoDrop ND-1000 spectrophotometer (Labtech). In addition, standard protocols were used to extract DNA from leukocytes.

2.3 | Next-generation sequencing

To study Nrf2 gene mutations in tumor specimens, single-nucleotide variants (SNVs), short insertions, and deletions (indels) were assessed by next-generation sequencing of the coding exons and intron flanking regions according to methods published elsewhere.[23] The associated custom primers were prepared with AmpliSeq Designer (Life Technologies), and the library was constructed and sequenced by an Ion AmpliSeq Library Kit 2.0, Ion PGM IC 200 kit, and Ion PGM (Life Technologies), in accordance with the manufacturer's instructions; these kits analyze a wide range of genes, including those recommended for analysis by the American College of Medical Genetics and Genomics.[24] Torrent Suite software were used to analyze the results of sequencing, and Torrent Variant Caller, Ion Reporter (v.5.1.0) was used for variant calling; Ion Torrent sequencer can accurately detect Nrf2 gene mutations.[25]

2.4 | Data analysis

Primary analysis of raw data was performed according to a previously published method.[23] In brief, after each sequencing, Torrent Suite version 4.2.1 evaluated signal processing, base calling, quality score assignment, adapter trimming, mapping to Genome Reference Consortium Human Build 37/Human Genome version 19, assessment of mapping quality, and variant calling. Then, a variant call file of the sequence variants (SNVs and indels) was created and examined on the online user interface. Integrative Genome viewer (Broad Institute) was used to visualize mapping and variant calling results.

						Nrf2	
H. Diffuse architecture	I. Necrosis	J. Venous invasion	K. Sinusoidal invasion	L. Capsular invasion	Weiss score	Nrf2 mutation	Nrf2 expression
0: absence	0: absence	0: absence	0: absence	0: absence			
1: presence	1: presence	1: presence	1: presence	1: presence			
				D+E+F+G+H+I+J+K+L;			
1	1	0	0	0	5	(-)	Low
1	1	1	1	1	9	c.1609G>A	High
1	1	1	1	1	8	(-)	High
0	1	1	0	1	3	(-)	Low
1	1	1	0	0	5	(-)	High
1	1	1	1	1	8	c.1346G>A	High
1	0	0	0	1	5	(-)	High
1	1	1	0	1	7	(-)	High
1	0	1	1	0	6	(-)	High
1	1	0	0	0	4	(-)	Low
1	1	1	1	1	8	c.70T>A	High
0	0	1	1	1	4	(-)	Low

2.5 | Immunohistochemistry

Sections of resected tumor tissue (4 μm thick) from the 21 patients were prepared by fixing in formalin and embedding in paraffin. Mouse monoclonal antibodies for Nrf2 (Abcam, ab-62352), Ki-67 (Abcam, ab-21700), p53 (Abcam, ab-131442), and β-catenin (Abcam, ab-16051) were used for staining.[23] Staining results were used to separate tumors into a group with low expression of anti-Nrf2, anti-Ki-67, anti-p53 antibody, and anti-β-catenin (<30% of cells with positive staining) and a group with high expression (≥30% of cells with positive staining).[23]

2.6 | Statistical analysis

Pearson's χ^2 test for contingency tables was used to analyze the association of Nrf2 expression with the Weiss score and Helsinki score. Two groups were compared by the Mann-Whitney *U* test, and three groups, by the Kruskal-Wallis test. Kaplan-Meier survival curves were generated and analyzed by log-rank test. Commercially available software was used for statistical analyses, and the significance level was set as a *P* value of less than 0.05. The study was performed in accordance with the Declaration of Helsinki. The Dokkyo Medical University Hospital Ethics Review Board approved the study protocol, and all participants provided written informed consent by using a form approved by the Dokkyo Medical University Institutional Committee on Human Rights in Research.

3 | RESULTS

The number of patients in each of the Weiss score and the Helsinki score subgroups is shown in Tables 1 and 2.

3.1 | Next-generation sequencing

Among the 12 ACCs, targeted next-generation sequencing of coding exons for Nrf2 gene identified three mutations in three out of the five ACCs with a Weiss score of 7 to 9. All mutations were on chromosome 2, as follows: 178095722, exon4, c.1609G>A, pGlu537Lys; 178095985, exon5, c.1346G>A, p. Arg449His; and 178098975, exon2, c.70T>A, p. Trp24Arg. In contrast, none of the seven ACCs with a Weiss score of 3 to 6 and none of the ACAs had an Nrf2 gene mutation ($p = 0.0244$, Table 1). By Helsinki scoring, only three ACCs with a Helsinki score of greater than 17 were Nrf2 mutants ($p < 0.0001$, Table 2).

3.2 | Immunohistochemistry for Nrf2 and its relation to SUVmax

Adrenocortical carcinomas with a Weiss score of 7 to 9 showed also a higher expression of Nrf2 ($p = 0.0056$), a higher expression of Ki-67 ($p = 0.0306$), and a higher baseline SUVmax ($p = 0.0003$) than ACCs with a Weiss score of 3 to 6 and ACAs (Table 1, Figures 1 and 2). Adrenocortical carcinomas with Nrf2 mutant had increased Nrf2 and Ki-67 expressions ($p = 0.0308$, $p = 0.0263$, respectively, Table 2, Figures 1 and 2) and an elevated baseline SUVmax ($p = 0.009$, Table 1). Furthermore, a higher Nrf2 expression was positively associated with a higher baseline SUVmax ($p = 0.0007$, Table 1), but not with a higher Ki-67 expression ($p = 0.1588$, Table 1).

Three ACCs with a Helsinki score of greater than 17 had also a higher expression of Nrf2 ($p = 0.0126$), a higher expression of Ki-67 ($p = 0.0235$), and a higher baseline SUVmax ($p = 0.0013$) than ACCs with a Helsinki score of less than 17 and ACAs (Table 2).

A higher expression of Ki-67 correlated with a higher baseline SUVmax ($p = 0.0367$, Table 2).

The expression of β-catenin was not related with Weiss score ($p = 0.1923$), Helsinki score ($p = 0.2691$), Nrf2 mutant ($p = 0.1147$), Nrf2 ($p = 0.6757$) and Ki-67 expressions ($p = 0.8991$), and SUVmax ($p = 0.3353$, Figure 3).

Higher expression of p53 was associated with an increased Weiss score ($p = 0.0037$), an elevated Helsinki score ($p = 0.0126$), Nrf2 mutant ($p = 0.0414$), a higher Nrf2 expression ($p = 0.0632$), and an increased SUVmax ($p = 0.0011$), but not Ki-67 expression ($p = 0.4884$, Figure 3).

3.3 | Survival

Four patients with ACC with a Weiss score of 7–9, including the three patients with Nrf2 mutant, died from the disease. Similarly, three patients with ACCs with a Helsinki score of greater than 17 and Nrf2 mutant died from the disease.

The comparison of ACCs with a Weiss score of 7–9 and those with a Weiss score of 3 to 6 found shorter overall survival in the group with a higher Weiss score ($p = 0.0429$, Figure 4A). Adrenocortical carcinomas with a Helsinki score of greater than 17 showed a poorer survival ($p < 0.05$, Figure 4B). Adrenocortical carcinomas with Nrf2 mutant tumors and higher Nrf2 expression had a worse prognosis ($p = 0.0231$, Figure 4C and $p < 0.05$, Figure 4D, respectively).

The median SUVmax was 5.7. We used this value to divide the patients into two groups: those with an SUVmax less than 5.7 and those with an SUVmax greater than or equal to 5.7. A comparison of these two groups found that higher SUVmax tended to be associated with worse prognosis ($p = 0.2904$, Figure 4E). Patients with ACCs with higher Ki-67 expression also had a worse prognosis than those with ACCs with lower Ki-67 expression ($p = 0.2335$, Figure 4F).

The higher expression of p53 showed better survival ($p = 0.0157$), while the higher expression of β-catenin had no impact on worse survival ($p = 0.1150$).

4 | DISCUSSION

Adrenocortical carcinoma is a highly aggressive malignancy, and recurrence is frequent in advanced ACC, even after complete resection.

TABLE 2 Relationship between gene mutation and protein expression of Nrf2, and FDG-PET

Adrenal cortical carcinoma ($n = 12$)/ adenoma ($n = 9$)	Nrf2 gene mutation			Nrf2 expression			Ki-67 expression			SUVmax (mean ± SD) in FDG-PET	
	No ($n = 18$)	Yes ($n = 3$)	*p* value	Lower ($n = 12$)	Higher ($n = 9$)	*p* value	Lower ($n = 14$)	Higher ($n = 7$)	*p* value		*p* value
Adenoma: Weiss <2 ($n = 9$)	9	0	0.02	8	1	0.01	8	1	0.05	3.9 ± 0.8	0
Adrenocortical carcinoma: Weiss >3 ($n = 12$)	9	3		4	8		6	6		11.4 ± 5.1	
Adenoma: Weiss <3 ($n = 9$)	9	0	0.0037	8	1	0.01	8	1	0.03	3.9 ± 0.8	0
Adrenocortical carcinoma: Weiss 3–6 ($n = 7$)	7	0		4	3		5	2		8.7 ± 4.5	
Adrenocortical carcinoma: Weiss 7–9 ($n = 5$)	2	3		0	5		1	4		15.2 ± 3.4	
Helsinki score: 0-17 ($n = 12$)	12	0	< 0.0001	10	2	0.126	10	2	0.0235	4.8 ± 2.2	0.0013
Helsinki score: 17-34 ($n = 6$)	6	0		2	4		4	2		10.5 ± 4.4	
Helsinki score: >34 ($n = 3$)	0	3		0	3		0	3		17.4 ± 2.3	
Nrf2 gene mutation											
No ($n = 18$)				12	6	0.03	14	4	0.03	6.9 ± 4.1	0.009
Yes ($n = 3$)				0	3		0	3		17.4 ± 2.3	
Nrf2 expression											
Lower (non to low) ($n = 12$)							10	2	0.16	4.8 ± 1.8	0.00
Higher (moderate to high) ($n = 9$)							4	5		12.8 ± 5.2	
Ki-67 expression											
Lower ($n = 14$)										6.3 ± 4.2	0.04
Higher ($n = 7$)										11.9 ± 5.8	

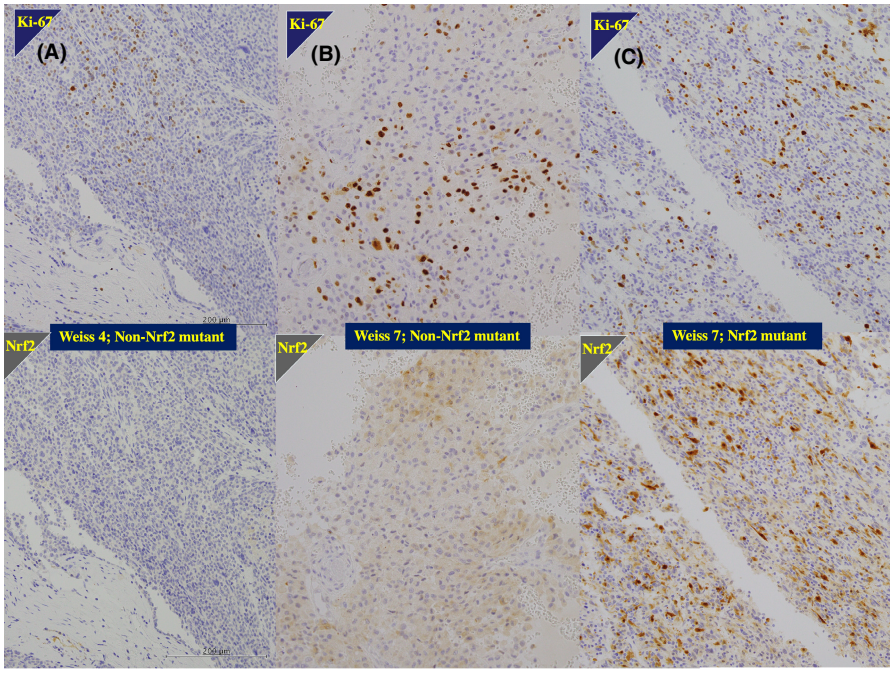

FIGURE 2 Immunohistochemistry of Ki-67 and Nrf2 in adrenocortical carcinoma (ACC). The same patients are presented in Figure 1. A, The tumor cells with a Weiss score of 4, a Helsinki score of 6.3, and without Nrf2 gene mutation showed lower Ki-67 labeling index (< 10%) and lower Nrf2 expression (<30%). B, The tumor cells with a Weiss score of 7, a Helsinki score of 11.7, and without Nrf2 gene mutation showed lower Ki-67 labeling index (<30%) and higher Nrf2 expression (≥30%), but weak intensity. C, The Nrf2 mutant tumor cells with a Weiss score of 7 and a Helsinki score of 19.3 displayed higher Ki-67 labeling index (≥30%) and higher Nrf2 expression (≥30%) with strong intensity

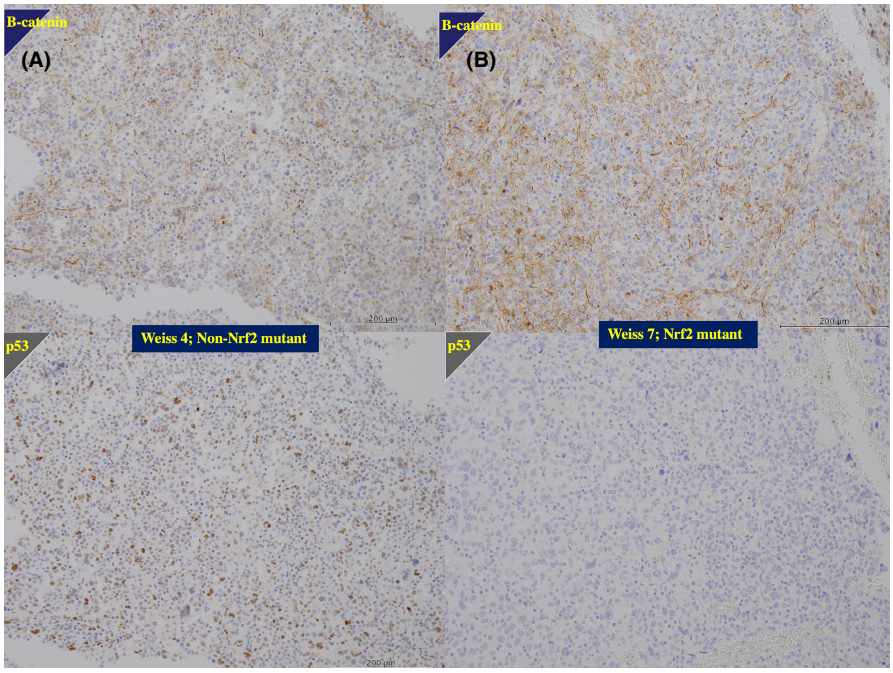

FIGURE 3 Immunohistochemistry of beta catenin and p53 in adrenocortical carcinoma (ACC). A, The same patient is presented in Figures 1A and 2A. Beta catenin and p53 were highly expressed in tumor cells. B, The same patient is presented in Figures 1C and 2C. Beta catenin was strongly expressed in tumor cells, but p53 was not expressed

After recurrence, the only treatment option is mitotane.[22] The ongoing phase III clinical trials Efficacy of Adjuvant Mitotane Treatment (ADIUVO) and ADIUVO-2 (www.adiuvo-trial.org) aim to further elucidate the efficacy of adjuvant mitotane in increasing disease-free survival in ACC. With the current treatments, the disease progresses in most patients, so there is an urgent need for new treatment approaches. In the present study in patients with ACC or ACA, we investigated from a metabolic perspective whether Nrf2 and SUVmax are associated with the Weiss criteria and the Helsinki scoring and showed that ACCs have higher Nrf2 expression and SUVmax than ACAs. Furthermore, patients with ACC—in particular ACC with a Weiss score of 7 to 9 and with a Helsinki score of greater than 17—with Nrf2 mutants have higher expression of Nrf2, a higher baseline SUVmax, and shorter overall survival. Our results indicate that activation of Nrf2 and elevated metabolism play roles in ACCs, and they highlight the importance of studying the roles of metabolic reprogramming, as well as the antioxidative stress response, in ACCs.

At present, various guidelines recommend using the Weiss criteria to determine whether tumors are benign or malignant.[7-9] Similarly, a Helsinki score appears to be a better scoring system.[10] However, because no sufficiently validated index exists, distinguishing between ACCs with more or less malignant potential is virtually

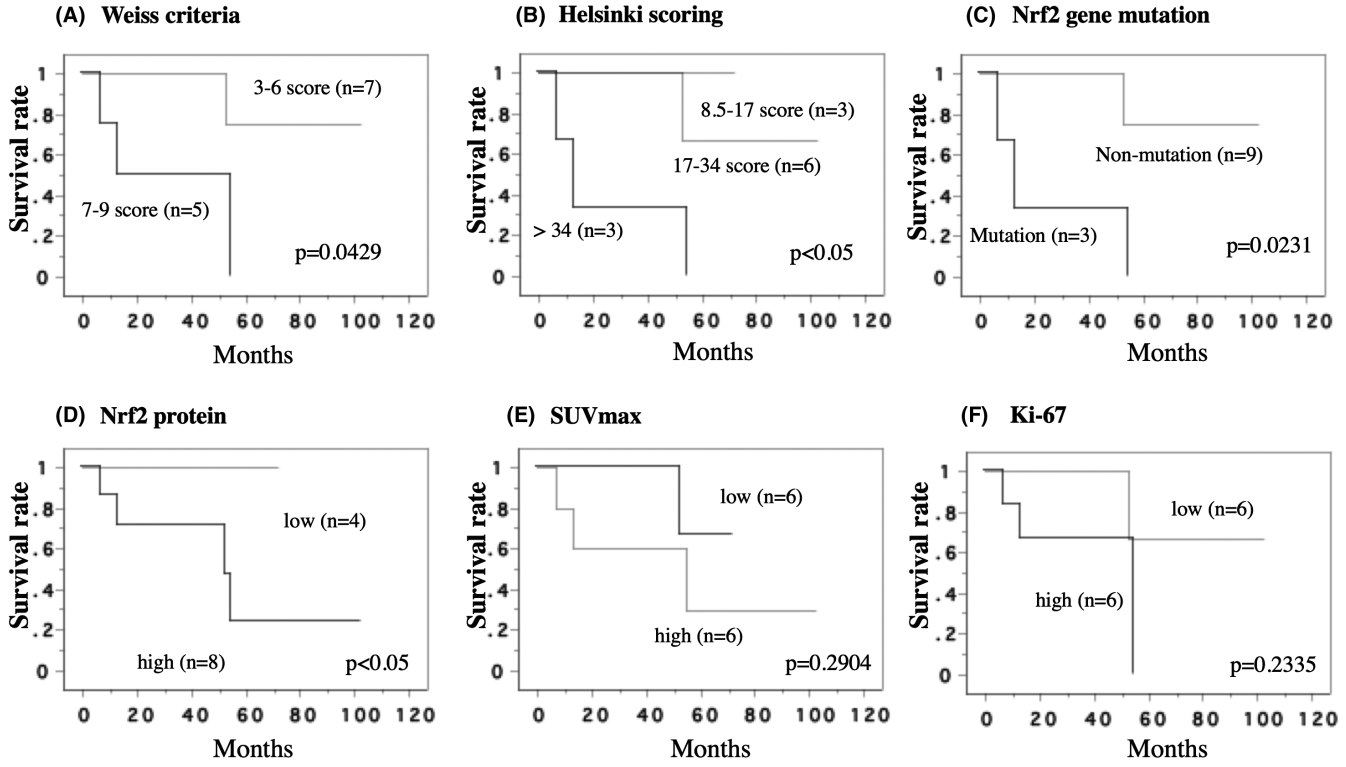

FIGURE 4 Overall survival curve. The survival curves reflect the Weiss criteria (A), Helsinki scoring (B), Nrf2 gene mutation (C), Nrf2 expression (D), SUVMax (E), and KI-67 in 12 ACCs (F). Adrenocortical carcinomas with higher Weiss score of 7-9 had shorter survival ($p = 0.0429$). Adrenocortical carcinomas with a Helsinki score with greater than 17 had shorter survival ($p < 0.05$). Adrenocortical carcinomas with Nrf2 gene mutation ($p = 0.0231$) and higher Nrf2 expression ($p < 0.05$) showed worse survival compared with those with non-Nrf2 mutation and lower Nrf2

impossible with sufficient sensitivity and specificity to be clinically useful, particularly in localized, resectable cases.

Cancer cells typically switch to glycolysis to generate adenosine triphosphate, and the associated increased glucose uptake represents a key change.[14,15] In addition to the well-documented cancer-preventive antioxidant function of Nrf2 signaling, many lines of evidence indicate that Nrf2 promotes various metabolic pathways and cell proliferation in cancers.[16-19] A high expression of Nrf2 was reported to be associated with poor prognosis in ACC,[20] and an increased accumulation of ^{18}F-FDG was seen in the disease.[21] However, to date few studies have investigated the relationship of Weiss criteria and Helsinki score with Nrf2 from a metabolic perspective. In the present study, we found an association between a Weiss score of 7 to 9 and a Helsinki score of greater than 34 and a mutation in the Nrf2 gene, higher Nrf2 expression, and higher SUVmax in the primary tumor. Moreover, Nrf2 mutant ACCs had higher expression of Nrf2 and higher SUVmax, and higher Nrf2 expression was positively associated with higher SUVmax, indicating that ACC might have metabolic characteristics. These findings suggest that Nrf2 activation might increase glucose uptake and be associated with more malignant behavior in ACC and that even if ACCs are localized and pathologically removed, those with a Weiss score of 7 to 9, a Helsinki score of greater than 34, and/or higher SUVmax will recur, necessitating active surveillance of patients.

We did not evaluate the molecular mechanism of overexpression of Nrf2 in ACCs. Other studies found that single-nucleotide polymorphisms (SNPs) in the Nrf2 promotor markedly decrease Nrf2 transcription and its activity. Some support was found also for a role of the SNP rs6721961 in the promoter region of the Nrf2 gene (Nrf2 regulatory SNP-617) in carcinogenesis.[26,27] A recent study found higher rates of lung cancer, especially among smokers, in people with the SNP rs6721961 in the antioxidant response element (ARE)-like sequence of the human Nrf2 promoter.[28] In that study, A/A homozygotes for the SNP rs6721961 had significantly (approximately 40%) lower Nrf2 mRNA levels than C/A heterozygotes and C/C homozygotes, as well as a higher risk of lung cancer. Thus, substitution of C with A at rs6721961 appears to significantly reduce Nrf2 mRNA expression. Furthermore, Nrf2 appears to be anticarcinogenic in humans. In contrast, our previous study in renal cell carcinoma showed that the prevalence of C/C was 60% in people with the SNP rs6721961, that of C/A was 34%, and that of A/A was 6% and that C/A and A/A were correlated with a higher expression of Nrf2 protein and shorter overall survival.[29] Thus, the role of this SNP in carcinogenesis and development of ACCs warrants additional investigation, and we are currently planning a larger study on this topic. On the other hand, initial reports of Nrf2 mutations in various cancers revealed that all the mutations clustered into the Neh2 domain, which is a critical site for

Kelch-like ECH-associated protein 1 (Keap1) binding.[30,31] In the current study, which revealed three Nrf2 gene mutations in ACC patients, one (p. Trp24Arg) was found at the Neh2 domain. Thus, this mutation disrupts Nrf2-Keap1 binding, and this mutant Nrf2 is not ubiquitinated and accumulates in nucleus. Other two mutations (p. Glu537Lys and p. Arg449His) were found at the Neh1 domain which is DNA and Maf, DNA binding proteins possessing a basic region-leucine zipper (bZip) motif, binding domain.[30,31] As Nrf2 mutations in the Neh1 domain in cancers have not been reported, mechanisms of Nrf2 upregulation in these mutants are unknown. It is possible that these mutations in the Neh1 domain stabilize Nrf2-Maf dimerization and/or Nrf2-Maf-ARE binding and consequently upregulate the Nrf2 pathway in these mutants. Nrf2 protein residues resulting from mutations of the Nrf2 gene interact with Keap1 to increase the activity of cap'n'collar-basic leucine zipper, a transcription factor.[16-19] Functional Keap1 mutations (missense, frame shift, and homozygous deletion) arise in a number of cancers, resulting in upregulation of Nrf2/ARE gene transcription.[16-19] Thus, additional research is required also to elucidate the roles of Keap1 in ACC. Recently, the relationship between the clinical features of ACCs and their genotyping has been widely studied, and large studies identified high chromosomal aneuploidy as one of the molecular characteristics of ACC and *CTNNB1* and *TP53* as the most commonly involved genes.[12,13] Furthermore, a gene expression profiles study by ENS@T used pan-molecular characterization of ACCs to separate tumors into two subgroups: C1A, that is, aggressive disease in which *CTNNB1* and *TP53* were prevalent and outcomes were worse; and C1B, that is, slower-growing tumors.[12] Analysis with the more comprehensive data set, The Cancer Genome Atlas, found three subgroups of ACC: Cluster of Cluster (CoC) I, with better outcome; CoC II, with intermediate outcome; and CoC III, with worse outcome.[13] In this study, though it is unclear how the Nrf2 mutation is incorporated into this classification, we found that the Nrf2 mutant in ACCs has poor outcomes. Furthermore, expression of β-catenin did not correlate with the Weiss and Helsinki scores, Nrf2 and Ki-67 expressions, SUVmax, and survival, while lower expression of p53 was associated with higher Weiss and Helsinki scores, Nrf2 mutation, increased Nrf2 expression, elevated SUVmax, and worse survival, indicating that signal crosstalk between Nrf2 and p53 should be studied in a forthcoming study. We should also examine gene analysis of TP53 and CTNNB1 in the future. Such analysis might lead to a useful risk classification in ACCs.

Following strategy could be proposed to target the Nrf2 signaling pathway for cancer therapy: (i) downregulation of Nrf2 at transcription level; (ii) increased degradation of Nrf2 mRNA; (iii) enhancement of Nrf2 degradation through E3 ubiquitin ligase complexes; (iv) inhibiting the dimerization of Nrf2 with small Maf proteins; and (v) blocking the binding of Nrf2-MAF to DNA.[32] Many small-molecule compounds with Nrf2-inhibitory activities have been reported in the literature; however, some of these inhibitory compounds in fact activate Nrf2 expression in other experimental settings and may have limited potential.[33] To date, none of the Nrf2-specific inhibitors have entered clinical trials for targeted cancer therapy. Further studies are warranted to identify more potent and specific Nrf2 inhibitors and clarify their mechanisms of action for treatment of patients with aggressive ACCs with Nrf2 overexpression.

Our study has several limitations, such as the retrospective design and the relatively small sample size. Furthermore, the maximum follow-up period of 10 years may not have been long enough to enable conclusions to be reached. Thus, a larger cohort should be tested to establish the role of Nrf2 signaling in ACC. Accessing the ACC data base might also be necessary to confirm our findings in a larger population in the future. In addition, we did not elucidate the molecular mechanism of Nrf2 activation in ACC; therefore, we plan to study the SNP rs6721961 in the Nrf2 gene promoter region, and—as mentioned above—mutation of Keap1, TP53, and CTNNB1 genes in ACC and the association of signal crosstalk between Nrf2 and other pathways.

In conclusion, our study found that patients with a Weiss score of 7 to 9, a Helsinki score of greater than 17, and Nrf2 mutant ACC have shorter overall survival, suggesting that activation of Nrf2 and the associated increase in metabolism play roles in ACC, in particular in more severe, malignant ACC. Large, prospective controlled trials are needed to confirm these results, with the ultimate aim to develop new treatments for ACCs.

ACKNOWLEDGEMENTS

The authors are especially grateful to Kin-ichi Matsuyama, Junka Hamano, and Aya Shimada for their excellent assistance in this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHORS CONTRIBUTIONS

Takao Kamai: Conception and design, development of methodology, acquisition of data, analysis and interpretation of data, writing of the manuscript, and study supervision. Satoshi Murakami, Kyoko Arai: Development of methodology, analysis and interpretation of data. Kazuyuki Ishida: Development of immunohistochemical study. Toshiki Kijima: Study supervision.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional ethics review board of Dokkyo Medical University Hospital (approval no. R-31-10J.). Before surgery, written consent regarding the use of surgical samples and blood as well as data publication was requested, and protection of patient privacy in future studies was guaranteed through a signed consent form approved by our institutional Committee on Human Rights in Research. Surviving patients provided written consent for analysis using the surgical samples, through a signed form approved by our institutional Committee on Human Rights in Research. All clinical samples were anonymized before analysis to guarantee protection

of patient privacy. Consequently, each patient in this cohort signed the consent form approved by our institutional Committee on Human Rights in Research (approval no. R-31-10J.).

CONSENT FOR PUBLICATION

Each patient signed an informed consent and subsequent publication form that was approved by our institutional Committee on Human Rights in Research. All clinical samples were anonymized before analysis to guarantee protection of patient privacy.

REGISTRY AND THE REGISTRATION NUMBER OF THE STUDY

Approval no. R-31-10J.

ANIMAL STUDIES

None.

ORCID

Takao Kamai https://orcid.org/0000-0002-7086-6964

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How to cite this article: Kamai T, Murakami S, Arai K, Ishida K, Kijima T. Association of Nrf2 expression and mutation with Weiss and Helsinki scores in adrenocortical carcinoma. *Cancer Sci*. 2022;113:2368–2377. doi:10.1111/cas.15379