European Review for Medical and Pharmacological Sciences 2018; 22: 3694-3702

Aberrant *NEK2* expression might be an independent predictor for poor recurrence-free survival and overall survival of skin cutaneous melanoma

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Abstract. – OBJECTIVE: Never in Mitosis (NIMA) Related Kinase 2 (Nek2) is a serine/threonine-protein kinase encoded by the NEK2 gene and is an essential enzyme in cell cycle progression. In this study, we investigated NEK2 expression profile, its independent prognostic value in terms of recurrence-free survival (RFS)/overall survival (OS), and the potential mechanisms of its dysregulation in melanoma.

PATIENTS AND METHODS: A retrospective study was conducted using data from Gene Expression Omnibus (GEO) datasets and the Cancer Genome Atlas (TCGA)-skin cutaneous melanoma (SKCM).

RESULTS: NEK2 was significantly upregulated in melanoma tissues. NEK2 upregulation independently predicted poor OS (HR: 1.500, 95% CI: 1.092-2.059, $p = 0.012$) and RFS (HR: 2.213, 95% CI: 1.298-3.772, $p = 0.004$). NEK2 DNA amplification was common in melanoma (192/366, 52.5%), which was associated with significantly elevated NEK2 expression. NEK2 expression was weakly and negatively correlated with its DNA methylation (Pearson's r = -0.29). Loss of p53 was associated with increased NEK2 expression.

CONCLUSIONS: Based on findings above, we infer that NEK2 expression independently predicts poor survival of melanoma. Its dysregulation might be related to DNA amplification/methylation and TP53 mutation.

Key Words:
NEK2, TP53, Melanoma, Overall survival, Recurrence-free survival.

Introduction

Never in Mitosis (NIMA) Related Kinase 2 (Nek2) is a serine/threonine-protein kinase encoded by the *NEK2* gene[1,2]. Nek2 plays an indispensable role in cell cycle progression, especially in mitotic regulation via phosphorylating different substrates. It participates in centrosome separation and bipolar spindle formation via phosphorylating centrosome related proteins such as CROCC, CEP250, and NINL[3,4]. It regulates kinetochore-microtubule attachment stability via phosphorylation of NDC80 during mitosis[5]. Besides, it also phosphorylates CDC20 and MAD2L1, thereby modulating the mitotic checkpoint protein complex[6].

Aberrant *NEK2* expression was observed in a wide variety of human cancers[7]. Its upregulation is associated with malignant transformation such as tumorigenesis, pathological development and drug resistance of multiple cancers, such as breast cancer[8], myeloma[9], and pancreatic cancer[10]. Inhibition of endogenous *NEK2* could significantly suppress cancer cell proliferation both *in-vitro* and *in-vivo* and has been considered as a potential therapeutic strategy[7,11,12]. Its expression is also considered as a promising biomarker for predicting poor prognosis in colorectal cancer[13], lung cancer[14], hepatocellular carcinoma[15], and ductal adenocarcinoma[16]. These findings suggest that *NEK2* expression might be a valuable biomarker for tumor progression and prognosis.

The mechanisms underlying *NEK2* dysregulation in cancers are quite complex and far from being fully understood. *NEK2* promoter activity might be suppressed by p53 in colon cancer cells[17]. Some miRNAs, such as miR-128 and miR-486-5p might also modulate *NEK2* translation[13,18]. However, whether other mechanisms are involved in its dysregulation are not clear.

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Currently, it remains a great challenge to identify melanoma patients at the highest risk of recurrence, which still highly relies on costly imaging studies[19-22]. In skin cutaneous melanoma, NEK2 acts as a potential driver of metastasis and is associated with drug resistance[23]. However, its expression profiles and its prognostic value has not been explored in melanoma. In this work, by performing a retrospective study using data in Gene Expression Omnibus (GEO) datasets and in the Cancer Genome Atlas (TCGA)-skin cutaneous melanoma (SKCM), we investigated *NEK2* expression profile, its independent prognostic value in terms of recurrence-free survival (RFS)/overall survival (OS) and the potential mechanisms of its dysregulation in melanoma.

Patients and Methods

This study was approved by the Ethics Committee of Linyi Central Hospital, China.

Data Mining in the GEO Datasets

NEK2 expression in normal skin and cutaneous malignant melanoma tissues were examined by data mining in the GEO datasets (https://www.ncbi.nlm.nih.gov/geo/) with GEO2R tool. We found one previous Affymetrix Hu133A microarray that compared gene expression in the transformation of melanocytes to melanomas (Reference Series: GSE3189)[24]. In this array, *NEK2* expression was examined in 45 primary melanoma, 18 benign skin nevi, and 7 normal skin tissues.

Data Mining in TCGA-SKCM

NEK2 RNA-seq data, as well as the clinicopathological data of patients with skin cutaneous melanoma in TCGA were obtained from the UCSC Xena Browser (https://xenabrowser.net/), which provides access to download the level 3 data in TCGA. Only the patients with primary (N=102)/metastatic melanoma (N=357) and intact OS data were included in survival analysis. Among these 549 cases, 295 cases had intact recurrence data were included in RFS analysis.

Clinicopathological data, including age at initial pathologic diagnosis, sample type, gender, pathological N status, pathological M status, pathological stages, clinical stage, Clark level value, ulceration indicator, Breslow depth value, radiation therapy, recurrence status, and overall survival status were downloaded.

NEK2 DNA methylation data (Illumina 450k infinium methylation beadchip) and Gene-level thresholded GISTIC2-processed copy-number data, which defines copy number alterations (CNAs) as homozygous deletion (-2), heterozygous loss (-1), copy-neutral (0), low-level copy gain (+1), high-level amplification (+2) were downloaded from the Xena browser.

Statistical Analysis

NEK2 expression in groups with different clinicopathological parameters was compared using one-way ANOVA followed by Tukey post-hoc test or using Welch's *t*-test. Receiver operating characteristic (ROC) analysis for death and recurrence detection was conducted to identify the best cutoff (Youden index) for *NEK2* expression. The association between clinicopathological parameters and *NEK2* expression was analyzed using chi-square tests. Kaplan-Meier curves of RFS and OS were generated by setting the Youden index as the cutoff, using GraphPad Prism 6.0 (GraphPad Inc., La Jolla, CA, USA). The difference between the survival curves was assessed using the Log-rank test. Univariate and multivariate Cox regression models were applied to evaluate the independent prognostic value of *NEK2*. Linear regression analysis was conducted to evaluate the correlation between *NEK2* expression and its DNA methylation. $p<0.05$ was considered statistically significant.

Results

NEK2 is Upregulated in Melanoma Than in Normal Skin Tissues

In the GEO datasets, we found an Affymetrix Hu133A microarray that compared gene expression in the transformation of melanocytes to melanomas (Reference Series: GSE3189)[24]. Based on RNA array data from 45 primary melanoma, 18 benign skin nevi, and 7 normal skin tissues, we found that *NEK2* was significantly upregulated in melanoma tissues compared with skin nevi and normal skin tissues (Figure 1A).

NEK2 is Significantly Upregulated in Recurrence and Death Groups

NEK2 has been characterized as an oncogene and is associated with drug resistance of melanoma[23,25]. By using clinicopathological data in TCGA-SKCM, we found that *NEK2* expression was significantly higher in recurrence and death

groups compared with the respective controls (p = 0.0076 and 0.0094, respectively) (Figure 1B-C).

High NEK2 Expression is an Independent Predictor of Poor OS and RFS in Melanoma

Then, we tried to assess the association between *NEK2* expression and survival outcomes. The patients were divided into high and low *NEK2* expression groups according to the best cutoff identified by ROC for death and recurrence detection. The association between clinicopathological parameters and high/low *NEK2* expression determined by ROC for death detection was summarized in Table I. Chi-square analysis showed that the high *NEK2* expression group had a significantly higher proportion of patients received radiotherapy (34/250, 13.6% *vs.* 15/208, 7.2%, p = 0.028) and death (140/250, 56.0% *vs.* 82/209, 38.3%, p = 0.0003) (Table I).

By generating Kaplan-Meier curves of OS and RFS, we found that high *NEK2* expression was associated with significantly worse OS (p = 0.0006) and RFS (p = 0.0002) (Figure 2A-B). Then, we performed univariate and multivariate analysis to assess the independent prognostic value of *NEK2* in terms of OS and RFS. In univariate analysis, increased age, primary melanoma, nodal invasion, distant metastasis, advanced pathological stages, high Clark level value, ulceration, increased Breslow depth value and high *NEK2* expression were associated with unfavorable OS (Table II). The following multivariate analysis indicated that high *NEK2* expression was an independent predictor of poor OS (HR: 1.500, 95%CI: 1.092-2.059, p = 0.012), even after adjustment of other significant variables in univariate analysis (Table II).

In terms of RFS, univariate analysis showed that increased age, primary melanoma, nodal invasion, distant metastasis, advanced pathological stages, high Clark level value, ulceration, increased Breslow depth value, and high *NEK2* expression were also associated with unfavorable RFS (Table III). Multivariate analysis revealed that high *NEK2* expression was an independent predictor of unfavorable RFS (HR: 2.213, 95%CI: 1.298-3.772, p = 0.004), even after adjustment of other significant variables in univariate analysis (Table III).

NEK2 DNA Amplification and Hypomethylation Are Related to Aberrant NEK2 Expression in Melanoma

Then, we examined the potential mechanisms underlying dysregulated *NEK2* in melanoma. Among 366 patients with copy number altera-

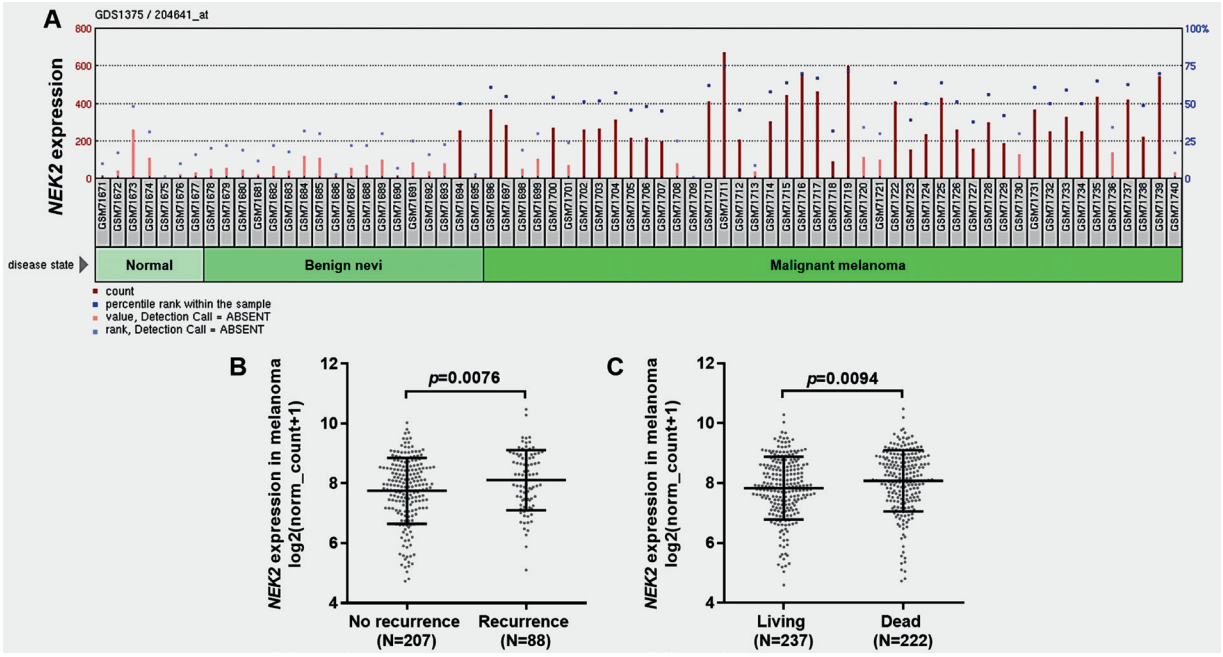

Figure 1. ***NEK2* was significantly upregulated in melanoma than in normal skin tissues.** Comparison of *NEK2* expression in 45 primary melanoma, 18 benign skin nevi, and 7 normal skin tissues. Data can be accessed via: https://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS1375:204641_at. ***B-C***, Comparison of *NEK2* expression in patients with or without reoccurrence (**B**) and between living and dead patients ***(C)***.

Table I. The association between clinicopathological parameters and NEK2 expression.

		NEK2 expression			
Parameters		**High (N = 250)**	**Low (N = 209)**	χ^2	***p*-value**
Age (continuous, mean ± SD)		58.83 ± 16.10	57.22 ± 15.26		0.28
Sample	Metastatic	202	155	2.9	0.089
	Primary Tumor	48	54		
Gender	Female	83	91	5.17	0.02
	Male	167	118		
Pathological N	N0	126	103	0.073	0.79
	N1+	95	82		
	NX + no data	29	24		
Pathological M	M0	225	184	0.0065	0.94
	M1+	13	11		
	no data	12	14		
Pathological stages	I/II	125	100	0.0013	0.97
	III/IV	107	85		
	no data	18	24		
Clark level value	I/II/III	58	41	0.67	0.41
	IV/V	117	101		
	no data	75	67		
Ulceration indicator	No	83	62	0.18	0.67
	Yes	91	75		
	no data	76	72		
Breslow depth value (continuous, mean ± SD)		5.43 ± 9.89	5.83 ± 6.34		0.66
Radiation therapy	No	216	193	4.85	0.028
	Yes	34	15		
	no data	0	1		
Recurrence Status	Living	100	107	0.96	0.33
	Dead	48	40		
	no data	102	62		
Living Status	Living	110	127	12.81	0.0003
	Dead	140	82		

NX: Nearby (regional) lymph nodes cannot be assessed.

tion measured, 192 patients (52.5%) had *NEK2* amplification (+1/+2) (Figure 3A). *NEK2* amplification was also associated with elevated *NEK2* expression (Figure 3C). Then, we examined the correlation between *NEK2* expression and its DNA methylation (Figure 3B). 471 cases had both *NEK2* DNA methylation and RNA expression quantified. In Illumina 450k infinium methylation beadchip, the methylation status of 24 CpG sites in *NEK2* DNA was measured. Regression analysis demonstrated that *NEK2* expression had a weak negative correlation with its DNA methylation (Pearson's r = -0.29) (Figure 3D).

NEK2 Expression is Related to TP53 Status in Melanoma

One previous paper[17] showed that *NEK2* is a p53-repressed gene in colon cancer. In this study, we further examined whether this suppressive effect exists in melanoma. By generating a heat map

Table II. Univariate and multivariate analysis of OS.

Parameters	Univariate analysis				Multivariate analysis			
	p	HR	95%CI (lower/upper)		p	HR	95%CI (lower/upper)	
Age (continuous)	0.000	1.024	1.015	1.034	0.004	1.016	1.005	1.027
Gender								
Female vs. Male	0.304	0.863	0.651	1.143				
Sample type								
Metastatic vs. Primary	0.000	0.371	0.240	0.574	0.011	0.491	0.284	0.849
Pathological N								
N1+ vs. N0	0.000	1.750	1.312	2.334	0.124	2.131	0.812	5.596
Pathological M								
M1+ vs. M0	0.044	1.873	1.017	3.450	0.132	1.995	0.813	4.898
Pathological stages III/IV vs. I/II	0.000	1.719	1.292	2.287	0.865	0.919	0.347	2.436
Clark level value								
IV/V vs. I/II/III	0.000	2.047	1.447	2.895	0.134	1.350	0.912	1.999
Ulceration indicator								
No vs. Yes	0.000	0.489	0.353	0.676	0.056	0.699	0.484	1.010
Breslow depth value (continuous)	0.000	1.027	1.014	1.041	0.238	1.010	0.993	1.027
NEK2 expression								
High vs. Low	0.001	1.606	1.221	2.111	**0.012**	**1.500**	**1.092**	**2.059**

M1+: M1a/M1b/M1c; N1+: N1/N2/N3

Table III. Univariate and multivariate analysis of RFS.

Parameters	Univariate analysis				Multivariate analysis			
	p	HR	95%CI (lower/upper)		p	HR	95%CI (lower/upper)	
Age (continuous)	0.021	1.016	1.002	1.031	0.866	1.002	0.984	1.019
Gender								
Female vs. Male	0.097	0.669	0.416	1.076	0.478	0.811	0.455	1.446
Sample type								
Metastatic vs. Primary	0.000	0.290	0.157	0.536	0.006	0.317	0.139	0.724
Pathological N								
N1+ vs. N0	0.012	1.799	1.140	2.840	0.257	2.535	0.507	12.680
Pathological M								
M1+ vs. M0	0.045	2.218	1.017	4.835	0.068	3.030	0.921	9.965
Pathological stages III/IV vs. I/II	0.038	1.600	1.026	2.496	0.914	0.913	0.175	4.774
Clark level value								
IV/V vs. I/II/III	0.036	1.854	1.041	3.302	0.710	1.127	0.600	2.117
Ulceration indicator								
No vs. Yes	0.021	0.557	0.338	0.916	0.203	0.680	0.376	1.231
Breslow depth value (continuous)	0.002	1.030	1.011	1.049	0.568	1.008	0.981	1.037
NEK2 expression								
High vs. Low	0.000	2.245	1.460	3.452	**0.004**	**2.213**	**1.298**	**3.772**

M1+: M1a/M1b/M1c; N1+: N1/N2/N3

showing the correlation between *TP53* mutation (SNPs and small insertions and deletions (INDELs)) and *NEK2* expression in melanoma (Figure 4A), we found that the most of the mutations were non-silent *TP53* mutation (Figure 4A, dotted frame), which were associated with decreased *TP53* expression (Figure 4B). Interestingly, the non-silent *TP53* mutation group had significantly elevated *NEK2* expression (Figure 4A and C), suggesting that *NEK2* expression might also be regulated by p53 in melanoma.

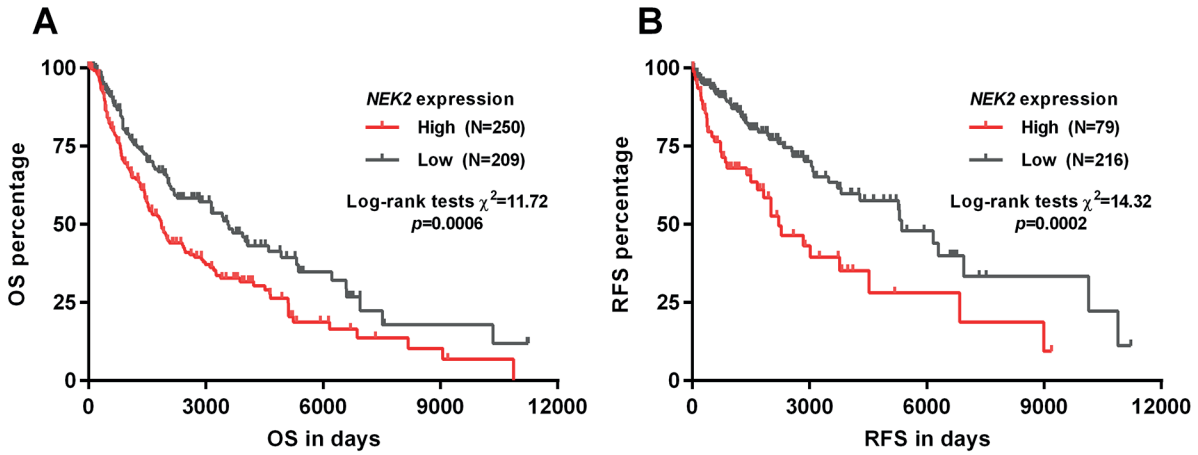

Figure 2. Kaplan-Meier curves of OS (A) and RFS (B) in skin melanoma. Patients were divided into high and low *NEK2* expression groups according to the best cutoff.

Discussion

An aberrant NEK2 expression is quite common in a wide variety of cancer cells. Since NEK2 has a critical role in the regulation of cell cycle progression, typically in centrosome separation, microtubule organization, chromatin condensation, and spindle assembly checkpoint, its upregulation directly leads to dysregulated cell cycle[7]. For example, enforced NEK2 expression in breast cancer MDA-MB-231 and MCF-7 cells results in centrosome amplification and multinucleation[26]. In patients with non-small cell lung cancer, Nek2 is a more efficient tumor proliferation marker compared with MCM7, and Ki-67[27]. In this study, by using data from one previous array, we confirmed that NEK2 was also upregulated in melanoma compared with normal skin tissues.

In recent years, some potential and promising biomarkers in multiple types of cancer were

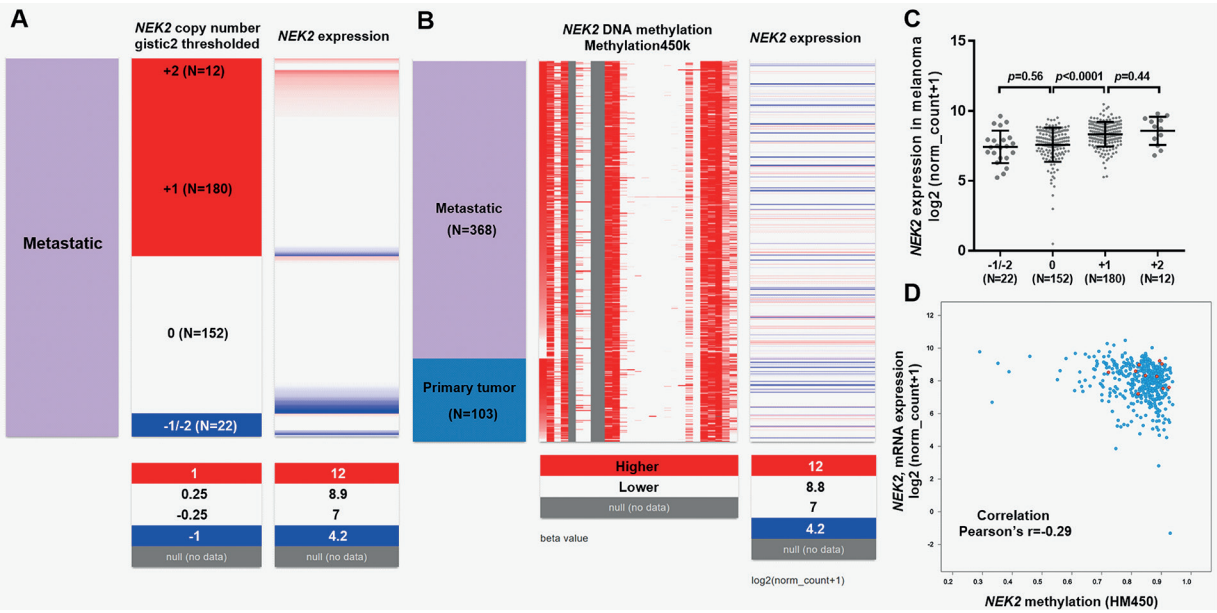

Figure 3. *NEK2* DNA amplification and hypomethylation are related to aberrant *NEK2* expression in melanoma. *A-B*, Heatmap showing the correlation between *NEK2* expression and its DNA amplification (A) and DNA methylation (B). -2: homozygous deletion; -1: heterozygous loss, 0: copy-neutral, +1: low-level copy gain, +2: high-level amplification.

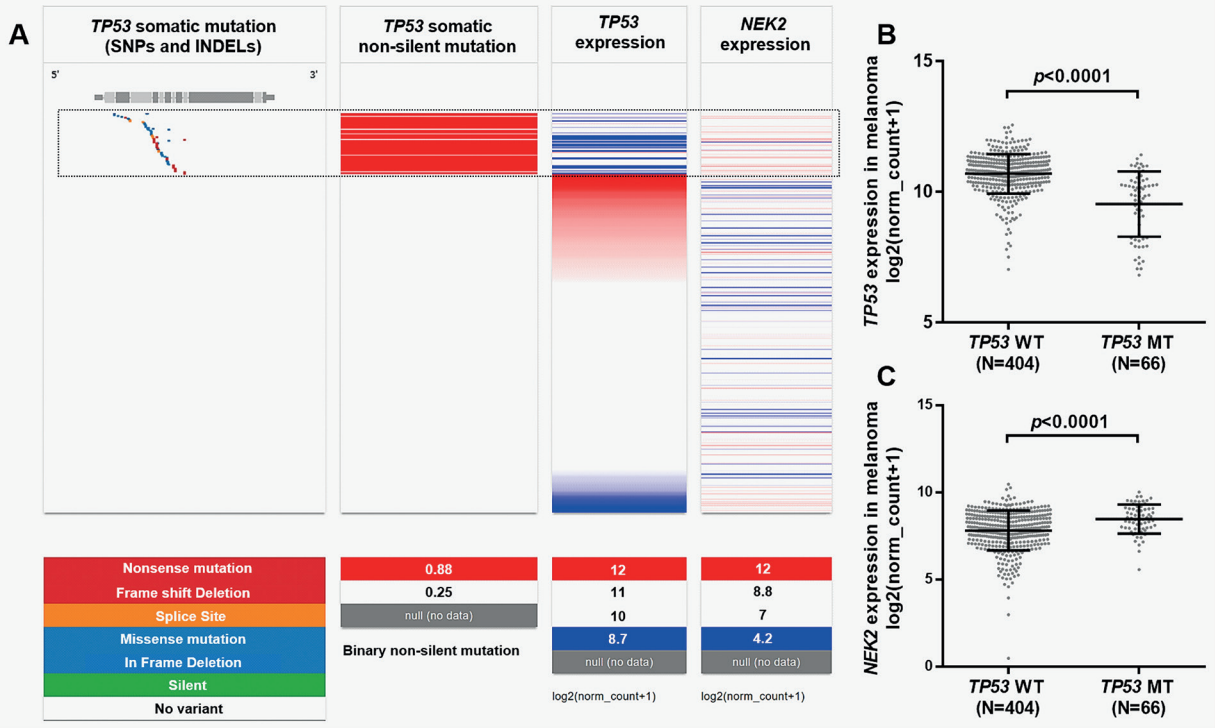

Figure 4. The correlation between *TP53* mutation and *NEK2* expression in melanoma. A, Heatmap showing the correlation between *TP53* mutation and *NEK2* expression in melanoma. **B-C,** Comparison of *TP53* (B) and *NEK2* (C) expression between *TP53* wild-type (WT) and non-silent mutation (MT) group.

identified by using data in TCGA. Zhou et al[28] compared the prognostic value of epithelial cell transforming 2 (ECT2) in lung squamous cell carcinoma and LUAD and found that increased ECT2 expression might serve as an independent prognostic biomarker of LUAD in terms of OS and RFS. PITX2 DNA methylation was identified as a prognostic biomarker in patients with head and neck squamous cell carcinoma (HNSC), by using data of HNSCC patients from TCGA[29]. In this study, by using data from TCGA-SKCM, we also confirmed significantly increased NEK2 expression in the death and recurrence groups compared with their respective controls. These findings suggest that NEK2 overexpression might also be related to worse survival outcomes in melanoma.

In fact, a series of previous studies observed that elevated NEK2 expression is usually correlated with malignant transformation and tumor progression. Its expression is also considered as a promising biomarker for predicting poor prognosis. In patients with colorectal cancer, the high NEK2 mRNA expression group had greater tumor depth, lymphatic invasion and peritoneal dissemination compared with the low NEK2 expression group[13]. NEK2 upregulation was associated with advanced tumor stage in patients with prostate cancer, lung cancer[14], and hepatocellular carcinoma[15]. With regard to overall survival (OS), NEK2 might be an independent prognostic indicator of poor OS in ductal adenocarcinoma[16], in non-small cell lung cancer[30], and colorectal cancer[13]. In this report, by generating Kaplan-Meier curves of OS and RFS, we showed that high NEK2 expression was associated with unfavorable OS and RFS. The following univariate and multivariate showed that high NEK2 expression was an independent predictor of poor OS and RFS, even after adjustment of other significant variables in univariate analysis. In multivariate analysis, the classical prognostic markers such as pathological stage and pathological N status even do not have this independent prognostic value. These findings suggest that NEK2 expression might be a promising prognostic biomarker in melanoma.

The mechanisms underlying genetic dysregulation in melanoma are largely unknown[31,32]. In this study, by using the deep-sequencing data in TCGA-SKCM, we examined the potential genetic and epigenetic alteration in NEK2 DNA. Results showed that DNA amplification was common in

melanoma (192/366, 52.5%), which was also associated with elevated NEK2 expression. NEK2 expression was also weakly and negatively correlated with its DNA methylation (Pearson's r = -0.29). These findings suggest that DNA amplification and hypomethylation might confer increased NEK2 expression in melanoma. One previous study[17] found that p53 can directly and specifically interact with the distal NEK2 promoter, thereby suppress NEK2 promoter activity and the following transcription in colon cancer. In this study, we found that the loss of p53 due to non-silent TP53 mutation was associated with increased NEK2 expression, suggesting that NEK2 might also be a p53-repressed gene in melanoma. However, we only assessed the correlation between their expressions, our future work will try to explore the molecular regulative network of NEK2 and its potential relation with p53 in melanoma.

Due to the critical regulative effect on tumor proliferation, NEK2 has been considered as a promising therapeutic target in cancer. Some previous studies have explored the potential therapeutic effect on reducing the impact of NEK2 by using RNA interference, blocking its ATP binding site and interfering its interactions with other proteins[7]. However, none of these strategies have been undergoing a clinical trial. Therefore, the development of NEK2 as a therapeutic target is still in the very early stage. In fact, as a serine/threonine-protein kinase, it may interact with many other partners and its oncogenic properties have not been fully understood. Thus, it is helpful to further define its interaction partners and reaction substrates for future drug discovery against NEK2.

Conclusions

We showed that an aberrant *NEK2* expression might be an independent predictor for poor RFS and OS of skin cutaneous melanoma. Its dysregulation might be related to DNA amplification/methylation and *TP53* mutation in melanoma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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