Low-level expression of necroptosis factors indicative of a poor prognosis of squamous cell carcinoma subtype of non-small-cell lung cancer

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Research article

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Abstract

Background

A programmed cell death pathway, necroptosis may synergize with DNA damage response (DDR) in opposing tumor progression. While our basic mechanistic understanding of necroptotic cell death advances rapidly, its prognostic implications have not been thoroughly examined in cancer.

Methods

We measured expression level of nine proteins involved in necroptosis and DDR in primary stage I non-small-cell lung carcinoma (NSCLC) samples from 394 patients using a tissue microarray.

Results

We find that low-level expression of the necroptosis markers RIPK3 and PELI1 is associated with increased risk of patient death and that high-level expression of the key DDR factor p53 enhances this risk. These effects appear to be specific to squamous cell carcinoma (SCC) subtype of NSCLC patients but not observable in non-SCC patients.

Conclusions

Low-level expression of such necroptosis factors as RIPK3 and PELI1 in combination with high-level expression of the DDR factor p53 can serve as a critical indicator in predicting survival of patients with stage I SCC.

Background

“Hallmarks” of cancer have not only advanced our understanding of cancer biology but also provided a basis for biomarker development [1]. Resistance to cell death, in particular, constitutes a crucial axis of the cancer hallmarks and indeed cancers develop diverse means to avoid “programmed” cell death [2]. Acquisition of the ability of cancer cells to evade a form of programmed cell death, apoptosis, which proceeds with a cascade of caspase activation, is best known as such a resistance to cancer cell death. Recent studies, however, have unveiled a qualitatively distinct, caspase-independent form of programmed cell death termed necroptosis, which like apoptosis would go awry in many cancers [3].

Genetic and biochemical analyses have established basic mechanisms by which necroptosis is regulated. We now know that engaging necroptosis critically requires the formation of a microfilament-like protein complex called necrosome. Phosphorylation in cis and trans between Receptor-Interacting Protein Kinases 1 and 3 (RIPK1 and RIPK3) is known to cause necrosome to assemble. The necrosome in

turn activates the pro-necroptotic kinase Mixed Lineage Kinase domain-Like protein (MLKL), leading to cell death by plasma membrane rupture as in the unprogrammed mode of cell death necrosis. The E3 ubiquitin ligase Pellino-1 (PELI1), on the other hand, appears to dampen the necrosome activity by ubiquitinylating RIPK3 and its subsequent targeting to proteosomal degradation [4, 5]. This suggests that availability of enough necrosome factors in cells would crucially decide whether to mount a proper necroptotic cell death.

Notwithstanding the rapid advance in basic mechanistic understanding of necroptosis, how it is related to survival of cancer patients remains less explored. Studies using preclinical models of select cancers such as leukemia and pancreatic cancer have elucidated the translational relevance of necroptosis [4, 6, 7]. Notably, several cultured cell and preclinical studies additionally suggested an association of necroptosis with DNA damage response (DDR), which is orchestrated by the “guardian of the genome” p53 [1, 2, 8, 9]. Since radiation therapy and chemotherapy are presumed to mount DDR in cancer cells yet cancers can develop a wide array of DDR resistance, the suggested association between necroptosis and DDR requires further examination in cancer patients [10–13].

Importantly, necroptotic factors appear to influence tumor progression differentially in a manner specific to individual tumor types [4, 6, 7]. We focus our investigation on lung cancer, which is relatively less represented in preclinical necroptosis studies yet accounts for a substantial proportion (~ 20%) of cancer-related deaths globally [14]. A major type of lung cancer, non-small-cell lung cancer (NSCLC) has a particularly grave prognosis albeit a slowly improving survival trend in recent years. Thus, development of NSCLC biomarkers would enable us to detect the disease at a preclinical stage, to predict treatment outcome more accurately, and to employ better targeted drugs [15, 16]. Development of prognostic biomarkers for early-stage NSCLC is another interest of research in this field because the approximate value for the 5-year survival rate is a disappointing 70% [17]. Importantly, current adjuvant treatment does not confer a survival advantage and treatment decisions are not guided by biomarker [9]. Squamous cell carcinoma (SCC) constitutes an aggressive subtype of NSCLC, distinguishing itself from non-SCCs not only by distinct histology and genomic landscapes but also by unique clinical characteristics of age, smoking habit, and comorbid diseases [18, 19]. Given the significant differences between these subtypes, biomarkers specific to SCC are greatly needed.

To this end, we here investigated whether the expression of proteins involved in necroptosis and their combined expression with DDR proteins have any effects on the survival of patients with stage I NSCLC. We present data about the relationship between expression level of those proteins, NSCLC subtypes, and patient survival as follows.

Methods

Patients and tissue specimens

Consecutive patients with pathological stage I NSCLC who had been treated at the Inha University Hospital (Incheon, South Korea) were retrospectively included in the study (Supplementary Fig. 1).

Patients who underwent surgical tumor resection between 1 January 1997 and 31 December 2011 were initially considered for inclusion. The cancers were staged using the 7th edition Tumor-Node-Metastasis staging system [20]. Data from each patient who had been followed-up for at least 3 years after surgical resection and whose tumor had been completely resected (R0 resection) were included in the analysis. Data from patients who had undergone limited resection (segmentectomy or wedge resection), received any anticancer treatment before surgical resection, or had a history of other cancer were excluded.

Information about smoking habits, Eastern Cooperative Oncology Group performance status, CCIS, tumor size, T stage, lymphatic or vascular invasion (from the pathological specimens), type of surgery, and use of adjuvant chemotherapy was collected. To increase the quality of the information, radiological examination (e.g., chest computed tomography scan, positron-emission tomography) and surgical pathology reports, and clinical information, were reviewed independently for each patient. All formalin-fixed, paraffin-embedded (FFPE) blocks were sent to a laboratory (SuperBioChips, Seoul, South Korea) for tissue microarray (TMA) construction and immunohistochemical staining. Study protocol was approved by the institutional review board of the inha university hospital and informed consent requirement was waived.

Construction of tissue microarrays and immunohistochemistry

TMA construction from individual FFPE samples was performed as previously described [8]. Each tissue array block contained up to 50 specimens, which allowed all 394 specimens to be contained in 20 blocks. Commercially available antibodies were chosen for RIPK3, MLKL (EPR17514), and PELI1 (F-7); these proteins were chosen as they are known to play essential roles in necrosome formation and modulation (Supplementary Table 1) [3]. P53 (DO-7), gH2AX (JBW301), ATM (7C10D8), Chk2pT68, BRCA1pS1423, and ERCC1 (8F1) were chosen because of their well-defined roles in cellular responses to DNA damage [1, 8, 9].

Serial sections (4 mm) from the FFPE blocks were analyzed for protein expression by immunohistochemistry. All antibodies were tested using a human control TMA panel [21]. A pathologist performed the test procedure with manufacture's information and evaluated the quality of positive and negative controls in immunohistochemistry.

All immunohistochemical staining was performed with a BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ) and i-View detection kit (Ventana Medical System) by following a standard operating procedure at SuperBioChips Laboratory, Seoul, South Korea.

Evaluation of immunohistochemistry

TMA samples containing more than 500 malignant cells were considered for evaluation. Scanned images of the stained slides were viewed using Aperio ImageScope program (version 11.2.0.782; Aperio Technologies, Vista, CA, USA) at 20x objective magnification. Automated digital image analysis was performed using Genie classifier and Nuclear *v*9 algorithm (Aperio Technologies). The percentages of stained cells and intensities of staining were estimated in the cytoplasm for MLKL and PELI1 and in the nucleus for RIPK3, p53, gH2AX, ATM, Chk2, BRCA1, and ERCC1 by two pathologists (H.J.J. and L.K.). The evaluators were blinded to each patient's results for the clinical and pathological variables and survival status.

H-score method was applied to evaluate protein expression. The percentage of tumor cells with positive cytoplasmic or nucleus staining on each TMA core was calculated and assigned a score of either 0 (0% stained tumor cells), 0.1 (1-9%), 0.5 (10-49%), or 1 (≥ 50%). The staining intensity was assigned a score of either 0 (weakest intensity), 1, 2, or 3 (strongest intensity). Each H-score was obtained by multiplying the proportion by the staining intensity. The median values of all mean H-scores were used as the cut-off values for the classification of the expression of the nine proteins as either "low" or "high" (Supplementary Fig. 2).

Survival measurements

The overall survival as the endpoint of this study was calculated from the time of diagnosis to the time of the last follow-up or death due to any cause. Dates of death were obtained principally from review of the medical records. To increase the quality of the information regarding the survival status of patients lost to follow-up, missing information was either obtained by contacting the patients or their relatives by phone or mail (13 patients) or obtained from the Ministry of Public Administration and Security, South Korea (29 patients).

Statistical analysis

Distributions of the clinical variables and levels of protein expression between SCC and non-SCC groups were tested using either chi-square test or Mann-Whitney U test as appropriate as possible. Cox model was performed to estimate HR and its 95% CI for each clinical variable and protein. Proteins that were significant at 5% level in the simple Cox model were included in the multiple Cox model. Performance of the model was evaluated using Harrell's c-index. *P* values were corrected using the FDR for multiple comparisons [22]. Statistical significance was accepted when $p < 0.05$. Statistical analyses were performed using a statistical software package (SPSS version 19.0, SPSS, Chicago, IL).

Results

Characteristics of patients

Clinical characteristics of 394 patients with stage I NSCLC (197 with SCC, 175 with adenocarcinoma, and 22 with other histology) are presented in Table 1. The median age of the patients was 66 years. 160 patients (40.6%) have since died. The median survival time was 9.8 years. Each patient was classified into either SCC or non-SCC groups. The proportions of never smokers and women were greater in non-SCC (82% and 81% of the entire cohort, respectively, chi-square $p < 0.001$) than in SCC. Non-SCC group also had greater proportions of patients who were younger, had smaller tumors, lower T stages, and/or received a lobectomy.

Associations between survival and age, size of tumor, and T stage were statistically significant (Supplementary Table 2). Gender and Charlson comorbidity index score (CCIS), smoking habit, histology result, lymphatic or blood vessel invasion, type of surgery, and use of adjuvant chemotherapy were not statistically significant prognostic factors, however.

Expression of proteins involved in necroptosis or DDR

Expression levels of proteins (low versus high) were compared between the SCC and the non-SCC groups using a tissue microarray (Table 2). High-level expression of RIPK3, p53, gH2AX, ATM, Chk2, BRCA1, and ERCC1 was more common in SCC group than in non-SCC group. By contrast, low expression of MLKL and PELI1 was more common in SCC group than in non-SCC group.

Association of necroptosis and DDR proteins with patient survival

Analysis of the entire cohort for screening revealed that expression of neither necroptosis nor DDR proteins was significant for survival of the patients at the 5% level (Supplementary Table 3). However, histology results indicated that low expression of RIPK3, PELI1, or BRCA1, and high expression of p53 were significantly detected in the simple Cox proportional hazards model (Cox model) for SCC group patients ($p = 0.011$, 0.019, 0.029, and 0.016, respectively). However, the effects of the proteins were not apparent in the patients with non-SCC. In SCC group, the prognostic effect of expression of these four proteins were statistically significant after adjusting clinical or pathological variables [adjusted hazard ratio (aHR) (95% confidence interval (CI)), p value, and p value corrected by false discovery rate (FDR) (q value), 2.292 (1.242–4.228), 0.008, and 0.016, respectively, for low RIPK3; 2.007 (1.058–3.806), 0.033, and 0.033, for low PELI1; 2.555 (1.382–4.721), 0.003, and 0.012, for high p53; and 2.088 (1.133–3.850), 0.018, and 0.024, for low BRCA1] (Fig. 1 and Table 3). The estimated Harrell's c-index was 0.73 (0.69–0.77), indicating that the final model was accurate enough.

Combined effects of necroptosis and DDR protein expression on patient survival

Combined effects of a protein pair were analyzed to determine whether any combination of proteins involved in necroptosis and DDR pathway had a synergy on patient survival. We noted that the combination of RIPK3 with p53 potentiated the multiplicative effects in the patients with SCC (*p* value and *q* value, 0.001 and 0.005) (Fig. 2A and Supplementary Table 4). Patients with low RIPK3 and high p53 expression had markedly worse survival than patients with high RIPK3 and low p53 expression (aHR (95% CI), 8.394 (2.856–24.677)) (Fig. 2A and Supplementary Table 4). Similarly, the combination of PELI1 with p53 potentiated the multiplicative effects in patients with SCC (*p* value and *q* value, and aHR (95% CI), 0.007, 0.018, and 6.760 (2.173–21.024) for low PELI1 and high p53) (Fig. 2B and Supplementary Table 4). Combined effect of RIPK3 with BRCA1 was associated with survival of patients with SCC, but its significance was not observed (Supplementary Table 4).

Discussion

In summary, our analysis has found that expression of proteins involved in necroptosis, such as RIPK3, is a robust prognostic factor for patients with stage I SCC, but not for patients with non-SCC. We also note that the effect of the low expression of necroptotic factors such as RIPK3 on patient survival becomes more profound when the same patient displays a higher p53 expression.

Studies using cancer cell lines and animal models have found that RIPK3 has diverse effects on tumor progression [4, 6, 7]. Our data show that SCC patients with low RIPK3 expression have an approximately 2.3 times increased risk for death relative to those with high RIPK3 expression. This observation indicates that as found in patients with esophageal SCC, RIPK3 inhibits tumor progression in the patients with SCC [10]. Another necroptosis factor PELI1 also has diverse effects on survival in patients with diffuse large B cell lymphoma or melanoma [23, 24]. We found that SCC patients with low expression of PELI1 had an approximately 2.0 times increased risk for death when compared with those with high expression. Diversities of tumor types or tumor microenvironments likely account for these differences in the roles for RIPK3 and PELI1 in tumor progression.

Our data also reveal a synergistic effect of either RIPK3 or PELI1 when combined with p53. Patients with a low-RIPK3 and high-p53 expression had an approximately 8.4 times increased risk for death relative to those with a high-RIPK3 and low-p53 expression. Similarly, the combination of low PELI1 expression and high p53 expression increased the risk of death up to 6.7 times when compared with high PELI1 and low p53 expressions. Notably, a large proportion of patients (approximately 45%) had either a low-RIPK3 and high-p53 expression or a high-RIPK3 and low-p53 expression. This result suggests that the use of these potential biomarkers could considerably benefit the population of patients with SCC. The result that the combination of low RIPK3 and high p53 expression can promote tumor progression was expected because high expression of p53 indicates an abnormal function. RIPK3 or PELI1 may somehow interact with p53 in patients with SCC. In human fimbrial epithelium, RIPK3 function depends on the status of p53, and PELI1 can regulate p53 function by promoting cytoplasmic localization of MDMX [12, 24]. Furthermore, in this study, this relationship between RIPK3 or PELI1 and p53 was found. This issue merits further investigation using cell lines and preclinical models.

The results of this study should be interpreted with some caution. The results may be limited in generalization to other cohorts because we adopted a retrospective study design and they were not validated using an independent cohort. However, the sample size was large enough to evaluate the effects of protein expression. Our present study was designed to maintain the homogeneity of the study population. We used stringent criteria for inclusion or exclusion of patients' data to minimize selection bias. Information on clinical or pathological variables as potential confounders was comprehensively collected and extensively considered during the analysis. We did not provide a biological basis directly supporting the differential prognostic effects between the two histological groups. However, these findings are supported by previous mechanistic studies with cancer cell lines or animal models [4–7, 10–13, 24, 25], as well as differences in genomic architectures and clinical profiles between these groups [18, 19]. All in all, our findings demonstrate a clear translational relevance of decreased levels of select necroptosis factors in predicting a poor prognosis of SCC-subtype NSCLC.

Conclusions

This is the first study to demonstrate prognostic implications of necroptosis proteins and their association with expression of DDR proteins in patients with stage I NSCLC. Our results indicate that RIPK3 and PELI1 or their individual combination with p53 can help classify patients with SCC into groups of either low or high death risk, providing a novel insight to clinicians about which patients would require a thorough follow-up.

Supplementary Information

Additional file 1:

Supplementary Fig. 1. Study schema: patients' enrollment and immunohistochemistry.

Supplementary Fig. 2 Images for immunohistochemistry in proteins involved in necroptosis and DNA damage response. Expression of the proteins was classified into low and high by using a criterion of median value of all mean H-scores (H & E, x 200).

Supplementary Table 1 Survival of the patients with stage I NSCLC by clinicopathological variables.

Supplementary Table 2 Expression of proteins involved in necroptosis or DNA damage response and survival of the patients with stage I NSCLC according to histology.

Supplementary Table 3 Combined effects of proteins on survival of the patients with stage I squamous cell carcinoma.

Supplementary Table 4 Information on antibodies for proteins involved in necroptosis and DNA damage response.

Abbreviations

RIPK: Receptor-interacting protein kinase; MLKL: Mixed lineage kinase domain-like protein; PELI1: Pellino-1; DDR: DNA damage response; NSCLC: Non-small-cell lung cancer; SCC: Squamous cell carcinoma; FFPE: Formalin-fixed, paraffin-embedded; TMA: Tissue microarray; CCIS: Charlson comorbidity index score; HR: Hazard ratio; CI: Confidence interval.

Declarations

Acknowledgements

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Authors' contributions

J-S.R, J.H.L., S.O., and Y-S.K. conceived and designed the study. J-S.R., S.O., H-S.N., H-J.K., M.J.K., M.H.P., L.K., and Y-S.K. collected and analyzed the data. J-S.R., Y.J.S. and Y.S.K. performed the statistical analysis. J-S.R., H-S.N., Y.S.K., L.K., Y-J.H., Y.J.S. and J.H.L. interpreted the results. All authors wrote, reviewed and approved the manuscript including figures and tables.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of the Inha University Hospital and the informed consent requirement was waived.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

References

Table 1 Baseline characteristics of the patients with stage I NSCLC according to histology.

		Total (%)	SCC (%)	Non-SCC (%)	*P*
Age	Median (IQR)	66 (60—72)	67 (61—73)	65 (57—72)	0.017[a]
Gender	Men	291 (73.9)	177 (60.8)	114 (39.2)	<0.001
	Women	103 (26.1)	20 (19.4)	83 (80.6)	
Smoking habit	Never	99 (28.0)	18 (18.2)	81 (81.8)	<0.001
	Ever	254 (72.0)	150 (59.1)	104 (40.9)	
ECOG performance status	0—1	326 (97.0)	161 (49.4)	165 (50.6)	0.338
	2 or more	10 (3.0)	3 (30.0)	7 (70.0)	
CCIS	0	108 (35.8)	50 (46.3)	58 (53.7)	0.272
	1	162 (53.6)	82 (50.6)	80 (49.4)	
	2 or more	32 (10.6)	20 (62.5)	12 (37.5)	
Tumor size, cm	Median (IQR)	3 (2.3—4.2)	3.5 (2.5—4.5)	2.9 (2.2—4.0)	0.005[a]
T stage	1	147 (37.3)	60 (40.8)	87 (59.2)	0.007
	2	247 (62.7)	137 (55.5)	110 (44.5)	
Lymphatic invasion	No	274 (75.7)	137 (50.0)	137 (50.0)	0.625
	Yes	88 (24.3)	47 (53.4)	41 (46.6)	
Vascular invasion	No	267 (73.8)	133 (49.8)	134 (50.2)	0.551
	Yes	95 (26.2)	51 (53.7)	44 (46.3)	
Type of surgery	Lobectomy	324 (82.2)	152 (46.9)	172 (53.1)	0.004
	Bilobectomy	33 (8.4)	17 (51.5)	16 (48.5)	
	Pneumonectomy	37 (9.4)	28 (75.7)	9 (24.3)	
Adjuvant chemotherapy	No	346 (88.0)	174 (50.3)	172 (49.7)	0.878
	Yes	47 (12.0)	23 (48.9)	24 (51.1)	
MST	Years (95% CIs)	9.8 (7.9—11.8)	10.1 (7.4—12.8)	9.1 (NA)	0.671[b]
Death	No	234 (59.4)	118 (50.4)	116 (49.6)	0.918
	Yes	160 (40.6)	79 (49.4)	81 (50.6)	

p values obtained by chi-square test, except [a] Mann-Whitney U test, [b] log-rank test. SCC, squamous cell carcinoma; IQR, interquartile range; ECOG, Eastern Cooperative Oncology Group; CCIS, Charlson comorbidity index score; MST, median survival time; NA, not applicable.

Table 2 Expression of proteins involved in necroptosis and DNA damage response according to histology.

		SCC, n (%)	Non-SCC, n (%)	*P*
RIPK3	Low	89 (44.3)	112 (55.7)	0.026
	High	107 (56.0)	84 (44.0)	
MLKL	Low	115 (58.7)	81 (41.3)	0.001
	High	80 (41.0)	115 (59.0)	
PELI1	Low	116 (59.5)	79 (40.5)	<0.001
	High	79 (40.3)	117 (59.7)	
p53	Low	83 (42.3)	113 (57.7)	0.003
	High	112 (56.9)	83 (42.1)	
BRCA1	Low	102 (45.7)	121 (54.3)	0.067
	High	95 (55.6)	76 (44.4)	
gH2AX	Low	96 (43.4)	125 (56.6)	0.004
	High	101 (58.4)	72(41.6)	
ATM	Low	103 (46.4)	119 (53.6)	0.127
	High	94 (54.7)	78 (45.3)	
Chk2	Low	101 (40.4)	149 (59.6)	<0.001
	High	96 (67.1)	47 (32.9)	
ERCC1	Low	95 (41.3)	135 (58.7)	<0.001
	High	100 (61.7)	62 (38.3)	

Expression of the proteins was classified as low and high by using a criterion of median value of all mean H-scores. *p* values obtained by chi-square test. SCC, squamous cell carcinoma.

Table 3 Proteins involved in necroptosis and DNA damage response and survival of patients with stage I SCC: Multiple Cox model.

		aHR (95% CI)	*P*	*q*-value*
RIPK3	low *v* high	2.292 (1.242—4.228)	0.008	0.016
PELI1	low *v* high	2.007 (1.058—3.806)	0.033	0.033
p53	high *v* low	2.555 (1.382—4.721)	0.003	0.012
BRCA1	low *v* high	2.088 (1.133—3.850)	0.018	0.024

Expression of the proteins was classified as low and high by using a criterion of median value of all mean H-scores. *FDR corrected p values after adjustment for multiple comparisons. aHR, hazard ratio after adjusting for age, gender, smoking habit, Eastern Cooperative Oncology Group performance status, Charlson comorbidity Index score, tumor size, T stage, lymphatic or vascular invasion, type of surgery, and adjuvant chemotherapy.

Figures

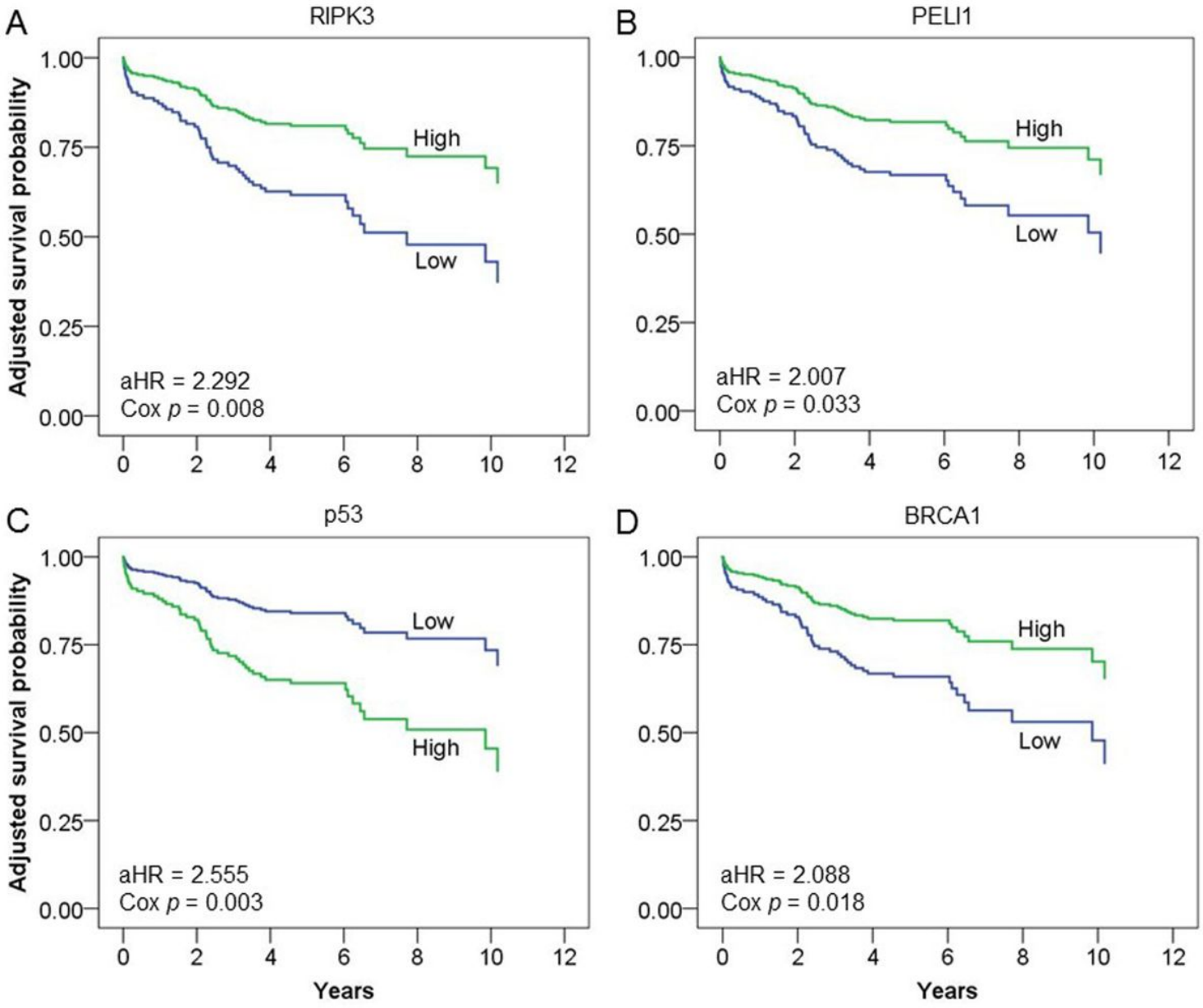

Figure 1

Survival of patients with stage I SCC according to the expression of RIPK3, PELI, p53 and BRCA1 in adjusted models. aHR: hazard ratio after adjusting age, gender, smoking habit, Eastern Cooperative Oncology Group performance status, Charlson comorbidity index score, tumor size, T stage, lymphatic or vascular invasion, type of surgery, and adjuvant chemotherapy.

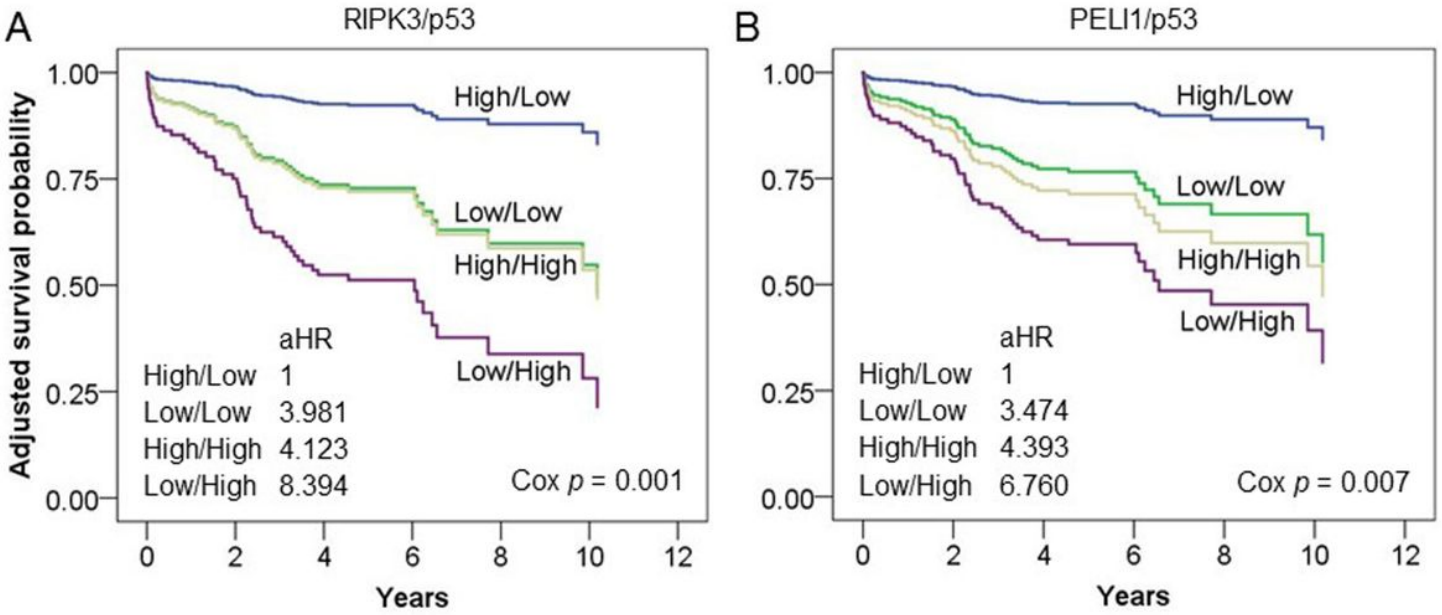

Figure 2

Combined effects of RIPK3 or PELI1 and p53 on the survival of SCC patients in adjusted models. aHR: hazard ratio after adjusting age, gender, smoking habit, Eastern Cooperative Oncology Group performance status, Charlson comorbidity index score, tumor size, T stage, lymphatic or vascular invasion, type of surgery, and adjuvant chemotherapy.

Supplementary Files

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