Lung Cancer 122 (2018) 131–137

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Overexpression of endoplasmic reticulum stress-related proteins, XBP1s and GRP78, predicts poor prognosis in pulmonary adenocarcinoma

Dohee Kwon[a], Jaemoon Koh[a,b], Sehui Kim[a], Heounjeong Go[c], Hye Sook Min[d], Young A Kim[e], Deog Kyeom Kim[f], Yoon Kyung Jeon[a,g,*,1], Doo Hyun Chung[a,b,*,1]

[a] Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, 03080, Republic of Korea
[b] Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, 03080, Republic of Korea
[c] Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, Republic of Korea
[d] Graduate School of Public Health, Seoul National University, Seoul, 08826, Republic of Korea
[e] Department of Pathology, Seoul Metropolitan Government-Seoul National University Boramae Medical Center, Seoul, 07061, Republic of Korea
[f] Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, Seoul Metropolitan Government-Seoul National University Boramae Medical Center, Seoul, 07061, Republic of Korea
[g] Seoul National University Cancer Research Institute, Seoul, 03080, Republic of Korea

ARTICLE INFO

Keywords:
Endoplasmic reticulum stress
GRP78
Non-small cell lung cancer
Unfolded protein response
XBP1 spliced form

ABSTRACT

Objectives: Endoplasmic reticulum (ER) stress is associated with tumor development and progression via pro-tumorigenic and anti-tumorigenic effects. However, the clinicopathological implications of the ER stress pathway in non-small cell lung cancer remain unclear. Therefore, we sought to address these issues in this study.
Materials and methods: Expression of two ER stress-related proteins, GRP78 and XBP1 spliced-form (XBP1s), was evaluated in pulmonary adenocarcinoma (pADC; n = 369) and squamous cell carcinoma (pSqCC; n = 246) using immunohistochemistry.
Results: Expression levels of GRP78 and XBP1s were significantly higher in pADCs and pSqCCs, respectively (both, $P < 0.0001$). In the pADC group, XBP1s expression was higher in patients with *ALK* translocation than in those with wild-type *ALK*, wild-type *EGFR*, or *EGFR* mutation ($P < 0.005$). No significant difference in GRP78 expression according to *ALK* or *EGFR* status was noted. pADC harboring high GRP78 expression exhibited an increased XBP1s expression ($P = 0.0067$). Higher XBP1s expression was associated with shorter disease-free survival (DFS) in patients with pADC ($P = 0.026$) and in those with *ALK* translocation ($P = 0.001$). Higher GRP78 expression was associated with shorter DFS in patients with pADC ($P = 0.029$) and those with *EGFR* mutation ($P = 0.005$). Multivariate survival analysis revealed that high XBP1s expression was an independent predictor of poor DFS in pADC ($P = 0.004$, hazard ratio [HR] = 3.115), and that high GRP78 expression was an independent predictor of poor DFS in *EGFR*-mutated pADC ($P = 0.007$, HR = 2.168). Taken together, high expression of XBP1s or GRP78 was an independent poor prognostic factor in pADC ($P = 0.002$, HR = 2.403).
Conclusion: GRP78 and XBP1s are expressed variably in pADC, but their overexpression is associated with poor patient prognosis. The ER stress pathway may be a prognostic biomarker and potential therapeutic target for pADC.

1. Introduction

The endoplasmic reticulum (ER) plays important roles in protein folding, post-translational modification, calcium homeostasis, and lipid biogenesis [1]. A variety of extrinsic and intrinsic stresses disturb ER homeostasis and elicit “ER stress,” leading to a series of cellular responses called “unfolded protein responses (UPRs)” [1,2]. UPRs have two functions: they increase ER folding capacity, ER-associated degradation of proteins, angiogenesis, autophagy, and antioxidant formation to maintain cell survival; and they induce apoptosis of cells when the ER stress is chronic and unresolved [1,3,4]. Three signaling pathways derived from ER-associated proteins (IRE1, PERK, and ATF6) mediate UPRs [1,2]. GRP78 is an ER chaperone protein that, under condition of ER stress, dissociates from IRE1, PERK, and ATF6 to initiate three unique signaling cascades [1,2,5,6]. Of the three ER stress pathways, IRE1 utilizes its RNase activity to degrade cellular RNAs

* Corresponding authors at: Department of Pathology, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul, 03080, Republic of Korea.
E-mail addresses: ykjeon@snu.ac.kr, junarplus@chol.com (Y.K. Jeon), doohyun@snu.ac.kr (D.H. Chung).
[1] Contributed equally to this work.

https://doi.org/10.1016/j.lungcan.2018.06.005
Received 11 January 2018; Received in revised form 30 May 2018; Accepted 8 June 2018

(termed IRE1-dependence decay) and decrease protein translation [1,5]. In addition, IRE1 splices the XBP1 mRNA to produce XBP1 spliced-form (XBP1s), which translocates into the nucleus and functions as a transcription factor for genes involved in the restoration of ER homeostasis [1,7,8]. Collectively, XBP1s and GRP78 are known to be essential proteins for the regulation of ER stress in various cells.

Previous studies have established that cancer cells are prone to induce ER stress due to nutritional deprivation, hypoxia, and oxidative stress. Oncogene activation or loss of the tumor suppressor gene also leads to ER stress in a cancer-cell intrinsic manner [1,9–13]. However, data on the functional roles of ER stress and UPRs in cancer cells have been conflicting. The cytoprotective tumor-promoting versus pro-apoptotic tumor-suppression effect of ER stress in tumor cells has been demonstrated, and these contrasting properties depend on cancer cell types, activated UPR arms, developmental stages of the tumor, and anti-cancer therapeutics [1,3]. Nevertheless, ER stress is accepted to be involved in the development and progression of cancer [1–3]. Therefore, an understanding of the direct correlation between ER stress and cancer biology might be critical for the prediction of clinical outcomes and development of therapeutic targets based on ER stress-related proteins.

The IRE1-XBP1 pathway plays important roles in tumor growth, metastasis, angiogenesis, and chemo-resistance in certain cancers [14–17]. For instance, among hematolymphoid malignancies, which exhibit variable IRE1-XBP1 activation, multiple myeloma has the highest XBP1s expression [18]. XBP1s is involved in the pathogenesis of multiple myeloma and is related to the clinical outcome [7,19–21]. The XBP1 pathway has been reported to be highly activated in triple-negative breast cancer compared with estrogen-receptor-positive breast cancer, and XBP1s has shown to interact with hypoxia-inducible factor (HIF)-1α to cooperatively activate HIF-1 target genes, thereby functioning as an oncogene in triple-negative breast cancer [22]. Taken together, these findings suggest that the roles of UPRs and the IRE1-XBP1 pathway differ among tumors based on the physiology and molecular genetic characteristics of cancer cells.

Non-small cell lung cancer (NSCLC) is a heterogeneous disease from pathological and genetic perspectives. Although molecular genetics-based combined therapies, including chemotherapy, targeted therapy, and immunotherapy, have improved the survival of patients with NSCLC, this disease remains the leading cause of death worldwide [23,24]. Thus, an understanding of a novel pathway in the pathogenesis of NSCLC is required for the development of new therapeutic approaches based on ER stress. However, little is known about the ER stress pathway in NSCLC. Thus, we investigated the expression of GRP78 and XBP1s and their prognostic implications in NSCLC according to molecular genetic features.

2. Materials and methods

2.1. Patients and samples

A total of 615 patients with resected NSCLC, including 369 patients with pulmonary adenocarcinoma (pADC) and 246 patients with pulmonary squamous cell carcinoma (pSqCC), were included in this study. All patients were of Korean descent, and underwent surgery and follow-up at the Seoul National University Hospital (SNUH). Patients with pADC consisted of those who consecutively underwent surgery from 2001 to 2011 with available formalin-fixed, paraffin-embedded (FFPE) tissue blocks, and those who underwent surgery for *ALK*-translocated pADC from 2012 to 2014. Patients with pSqCC were those who consecutively underwent surgery from 2004 to 2012 with available FFPE tissue blocks. No patient received neoadjuvant chemotherapy prior to surgery, and no distant metastasis was found at the time of diagnosis. The pathological tumor-node-metastasis (TNM) stage was classified based on the Cancer Staging Manual (7th edition) from the American Joint Committee on Cancer. A tissue microarray was constructed from 2-mm-diameter cores derived from representative tumor areas (i.e., predominant histological pattern in adenocarcinomas) of FFPE tissue blocks. For selected cases, normal lung tissues far from the tumor were included in TMA. This study was conducted in accordance with the recommendations of the World Medical Association Declaration of Helsinki and approved by the institutional review board of SNUH (IRB approval no. 1404-100-572).

2.2. Immunohistochemistry (IHC)

XBP1s expression was analyzed by IHC using a mouse anti-XBP1s (clone 143 F, active form) monoclonal antibody (Merck Millipore, Darmstadt, Germany). IHC was performed using the Benchmark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA) with antigen retrieval by CC1 buffer and an antibody dilution of 1:2000. The specificity of this antibody against XBP1s rather than XBP1 unspliced form was validated by western blotting (data not shown) and previous studies [18]. GRP78 IHC was performed using a rabbit anti-GRP78 polyclonal antibody (catalog no. ab21685; Abcam, Cambridge, UK) with an antibody dilution of 1:500.

The interpretation of IHC for XBP1s and GRP78 was performed manually in terms of staining intensity and the proportion of stained cells, by assessing the entire tumor core, with slight differences between the two proteins. XBP1s IHC staining was scored as 0 (no staining), 1 (weak to moderate staining), or 2 (strong staining), based on the intensity of nuclear expression, and the proportion (%) of the tumor cells with each score was determined. The H-score for XBP1s, which ranged from 0 to 200, was estimated using the following formula: (1 × percentage of cells with weak to moderate staining [i.e., score 1]) + (2 × percentage of cells with strong staining [i.e., score 2]). In contrast, GRP78 expression was observed in the cytoplasm and scored based on the intensity of expression (0, negative; 1, weak; 2, moderate; 3, strong staining) and the proportion of cells with each intensity. The H-score for GRP78, which ranged from 0 to 300, was calculated using the following formula: (1 × percentage of cells with weak staining) + (2 × percentage of cells with moderate staining) + (3 × percentage of cells with strong staining). One pathologist (D.K.) assessed the all IHC slides at three different time points blinded to the previous data, and then for the discrepant cases consensus scoring was made by evaluating the all IHC slides together with another pathologist (Y.K.J.).

To determine the statistically optimal cutoff value, various XBP1s and GRP78 expression data, including staining intensity, proportions of expressing cells, and H-scores, were inserted in the web-based program “Cutoff finder”, which is available online at http://molpath.charite.de/cutoff/ [25]. Specifically, we used “significance of correlation with binary variable” method with “recurrence or not during follow-up periods” as an outcome variable. Consequently, XBP1s expression was determined to be high when the proportion of cells with strong intensity exceeded 80%. In contrast, GRP78 expression was considered to be high when the H-score of the tumor exceeded 285.

2.3. Screening for major driver oncogene alteration events

Mutations of *EGFR* (exons 18, 19, 20 and 21) and *KRAS* (exons 2 and 3) were examined by direct Sanger sequencing or peptide nucleic acid (PNA)-clamping real-time PCR, and *ALK* translocation was examined by dual-color break-apart FISH analysis, as described previously [26].

2.4. Statistical analysis

Statistical analysis was performed using SPSS version 21.0 (IBM Corporation, Armonk, NY, USA). Associations between categorical data were assessed using Pearson’s χ^2 test, Fisher’s exact test as necessary. In addition, linear by linear association was analyzed for defining trends among multiple categorical variables. Meanwhile, continuous data was analyzed with Student’s *t*-test or one-way analysis of variants (ANOVA) method. Disease-free survival (DFS) was defined as the period between