Annals of Diagnostic Pathology 24 (2016) 47–51

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

Expression analysis of BRUCE protein in esophageal squamous cell carcinoma

Somayeh Salehi, MSc [a], Amir Hossein Jafarian, PhD [b], Mohammad Mahdi Forghanifard, PhD [a,*]

[a] Department of Biology, Damghan Branch, Islamic Azad University, Damghan, Iran
[b] Cancer Molecular Research Center, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

ARTICLE INFO

Keywords:
Bruce
IAP
Esophageal squamous cell carcinoma
Immunohistochemistry
Immunocytochemistry
Apoptosis

ABSTRACT

Apoptosis is a form of cell death in response to diverse stressful physiological or pathological stimuli. One of the most important gene families involved in apoptosis is inhibitors of apoptosis. As a member of inhibitors of apoptosis, BRUCE can suppress apoptosis and promote cell division. Because esophageal squamous cell carcinoma (ESCC) cells, as well as other cancer cells, are immortal, our aim in this study was to analyze BRUCE protein expression in ESCC and evaluate its correlation with tumoral clinicopathologic features. Fifty ESCC specimens were examined for BRUCE protein expression using immunohistochemistry. A defined scoring method was applied. BRUCE protein was detected in 82% of tumors. Tumor progression stage and invasion depth correlated significantly with BRUCE protein expression ($P = .019$ and .005, respectively). Furthermore, association of BRUCE expression with tumor location was near significant ($P = .058$). The correlation of BRUCE overexpression in ESCC and disease aggressiveness may confirm the importance of BRUCE in ESCC progression and invasiveness. Therefore, BRUCE protein may be a molecular marker for aggressive ESCC and, thus, a potential therapeutic target to inhibit tumor cell progression and invasion.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) remains a disease with poor prognosis despite improved surgical techniques and perioperative cancer management, which have improved survival rates to some extent [1]. The symptoms appear when the disease has already progressed to advanced stages III and IV. Moreover, ESCC cells develop resistance to chemotherapeutic drugs, resulting in dramatic decreases in 5-year survival rates for ESCC patients undergoing chemotherapy. A better understanding of the molecular mechanisms in ESCC carcinogenesis and progression would likely help to improve ESCC patients' prognoses. Therefore, identification of novel noninvasive biomarkers for early tumor discovery is greatly needed [2].

The parity between cell death and survival is one of the basic features of cellular homeostasis [3]. Programmed cell death is also beneficial for prevention of tumors and the spread of infectious diseases, as it eliminates damaged and infected cells, and cells that harbor harmful mutations. The inhibitor of apoptosis (IAP) proteins are involved in cell death, immunity, inflammation, cell cycle regulation, and migration [4]. IAP proteins are potent suppressors of apoptosis with 8 members, which include NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), BRUCE (Apollon, BIRC6), Livin/ML-IAP (BIRC7), and IAP-like protein 2 (BIRC8) [5]. The BRUCE protein is unusually large, at 528 kDa. It consists of single N-terminal baculovirus IAP repeat and C-terminal ubiquitin-conjugating domains. The latter has either chimeric E2/E3 ubiquitin ligase or antiapoptotic activity [6]. BRUCE can bind and inhibit caspases 3, 6, 7, and 9 [6], and facilitates proteasomal degradation of proapoptotic proteins such as caspase-9 [7], SMAC/DIABLO [7,8], and HTRA2/OMI [6,9], through its baculovirus IAP repeat and ubiquitin-conjugating domains, respectively. BRUCE is a critical regulator of cytokinesis and plays an important role in cell proliferation [10]. Recent studies support a role for BRUCE in conferring apoptosis resistance to cancer cells. BRUCE protein expression has been detected in a variety of cancer cell lines, and in vitro studies have shown that BRUCE functions as an apoptosis inhibitor in glioma (SNB-78) [11], lung cancer (H460) [12], cervical cancer (Hela) [8,10,13,14], fibrosarcoma (HT-1080) [7,14], osteosarcoma (U2OS) [10], and breast cancer cells (MCF-7 [14], ZR75.1, MDA-MB-231 [15]). In breast and lung cancer cells, loss of BRUCE expression has been demonstrated to trigger apoptosis through p53 stabilization and caspase-3 activation [12,15]. BRUCE expression has been also observed in colorectal cancer [16] and childhood de novo acute myeloid leukemia [17].

Although IAP protein expression has been studied in various malignancies, BRUCE expression and its potential clinical relevance have not been investigated in ESCC. Therefore, our aim in this study was to elucidate BRUCE protein expression in ESCC and its association

* Corresponding author at: Cell and Molecular Biology, Department of Biology, Damghan Branch, Islamic Azad University, PO Box 3671639998, Cheshmeh-Ali Blvd, Sa'dei Square, Damghan, Islamic Republic of Iran. Tel.: +98 912 711 6027.
E-mail addresses: forghanifard@gmail.com, forghanifard@damghaniau.ac.ir (M.M. Forghanifard).

http://dx.doi.org/10.1016/j.anndiagpath.2016.07.003
1092-9134/

with tumoral clinicopathologic features of the disease in ESCC patients who had undergone surgical resections.

2. Materials and methods

2.1. ESCC patients

Tissue samples were collected from 50 ESCC patients from Ghaem and Omid Hospitals of Mashhad University of Medical Sciences, Mashhad, Iran, who had undergone esophagectomy between 2011 and 2013. The recruited patients received no chemotherapy or radiotherapy before the surgery. All specimens were examined histologically by pathologists and their clinicopathologic features were determined according to the TNM classification system of the International Union Against Cancer [18]. Three sections from each tumor sample were selected for immunohistochemical (IHC) evaluation.

2.2. Immunohistochemistry

Esophageal squamous cell carcinoma and marginal normal tissues were analyzed for BRUCE protein expression. Paraffin-embedded tumor specimens fixed in neutral-buffered formalin were sectioned (4 μm) and placed on poly-L-lysine–coated glass slides. After antigen recovery at 94°C for 30 minutes, the sections were washed with distilled water and incubated with 0.3% H_2O_2 solution in methanol for 10 minutes to block endogenous peroxidases. The slides were then washed with tris buffer and incubated with protein block for 30 minutes to inhibit nonspecific binding. The slides were incubated with the primary antibody (antihuman BRUCE antibody #ab140200; Abcam, Cambridge, United Kingdom) for 60 minutes at room temperature. After washing, sections were treated with a secondary antibody (Sigma, Taufkirchen, Germany; RE7111). Sections were incubated with Novolink polymer for 30 minutes. The immunoreaction was visualized by adding 3,3′-diaminobenzidine for 10 minutes (RE7107). Finally, sections were counterstained with hematoxylin. Duodenal carcinoma tissue was provided as a positive control for immunostaining. Staining results were scored as described before [19].

2.3. Cell culture

The human ESCC cell line KYSE-30 was purchased from the Pasteur Institute (Tehran, Iran). KYSE-30 cells were grown in RPMI-1640 medium (Gibco, BRL Life Technology, Gaithersburg, MD) supplemented with 10% (vol/vol) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 95% humidified atmosphere and 5% CO_2.

2.4. Immunocytochemistry

To verify the results of the IHC and qualitative analyses of BRUCE protein expression, immunocytochemistry technique was applied on KYSE-30 cells. The fresh cultivated cells were fixed on slides and stained according to the IHC method.

2.5. Statistical analysis

The Statistical Package for the Social Sciences software version 11.5 (SPSS Inc, Chicago, Illinois) was used for statistical analyses. Either the χ^2 test or Fisher exact test was used to analyze the correlation between BRUCE expression and the tumors' clinicopathologic features. The correlations between different levels of protein expression and categorical data were analyzed using sample *t* test and analysis of variance. *P* values less than .05 were considered statistically significant.

3. Results

Immunohistochemistry and immunocytochemistry studies were performed on 50 surgical ESCC specimens and KYSE-30 cells, respectively. The clinicopathologic characteristics of the patients are summarized in Table 1. The patients' mean age (±SD) was 61.04 (± 12.52) years and ranged from 27 to 87 years. The study included 21 male and 29 female patients. The tumor sample mean size was 4.4 cm ± 2.1 cm and ranged from 1.50 to 12 cm. Tumors were resected from middle or lower parts of esophagus.

3.1. BRUCE protein expression and its clinical relevance

Immunohistochemical analysis of BRUCE protein expression was performed on ESCC and normal esophageal squamous tissues. Forty-one (82%) of ESCC tissues expressed BRUCE protein (Table 1). Based on the immunoreactivity scoring (IS) system, 7 (14%) tumor tissues showed strong BRUCE immunostaining, whereas 29 (58%) and 5 (10%) tumor tissues showed moderate and low BRUCE immunostaining, respectively. Nine (18%) tumor tissue samples were negative for BRUCE expression. Normal esophageal tissues showed less BRUCE expression than did ESCC tissues. Indeed, 25 (50%) of the normal tissues were negative for BRUCE protein expression, whereas 12 (24%) and 13 (26%) normal samples showed moderate and low BRUCE expression, respectively. Strong BRUCE protein immunostaining was not detected

Table 1
Clinicopathologic features of the ESCC tumors and their correlations with BRUCE protein expression

		Tissue immune staining						*P*
		Tumor			Normal			
		Negative	Low	High	Negative	Low	High	
Sex	Male	2	14	5	9	12	0	.222
	Female	7	17	5	16	13	0	
Node metastasis	No metastasis	5	28	8	18	23	0	.147
	Node metastasis	4	3	2	7	2	0	
Depth of tumor invasion	T1,2	1	9	2	8	4	0	**.005***
	T3,4	8	22	8	17	21	0	
Stage of tumor progression	Stage I/II	3	25	5	14	19	0	**.019***
	Stage III/IV	6	6	5	11	6	0	
Grade of tumor differentiation	PD	0	4	1	2	3	0	.321
	MD	8	25	8	20	21	0	
	WD	1	2	1	3	1	0	
Tumor location	Lower	5	9	2	8	8	0	.058
	Middle	4	20	7	16	15	0	
	Upper	0	2	1	1	2	0	

Abbreviations: MD, moderately differentiated; PD, poorly differentiated; WD, well differentiated.
* Statistically significant.