Bcl-2 and Noxa are potential prognostic indicators for patients with gastroenteropancreatic neuroendocrine neoplasms

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Abstract

Purpose Bcl-2 family proteins are of great significance in the pathogenesis and development of tumors. In this study, the correlations between the expression of Bcl-2 family proteins and clinicopathological features and prognosis of neuroendocrine neoplasms (NENs) were further investigated.

Methods 105 Patients diagnosed with gastroenteropancreatic NENs (GEP-NENs) with the paraffin specimen of the tumor available were retrospectively included. Immunohistochemistry (IHC) was performed to detect the expression of Bcl-2 family proteins in paraffin-embedded samples. Student’s t-test and Chi-square test were applied to compare the difference of quantitative and categorical variables, respectively. Survival analysis was conducted according to Kaplan–Meier method. Univariate and multivariate cox regression analysis were used to identify the independent prognostic factors.

Results The IHC score of Bcl-2 was significantly higher in neuroendocrine carcinoma (NEC) patients (65.6%), while a higher IHC score of Noxa was more common in neuroendocrine tumor (NET) patients (49.3%). Survival analysis indicated that patients with higher Bcl-2 expression and lower Noxa expression had worse 5-year survival (39.3% vs. 75.6%, \( p < 0.001 \); 40.6% vs. 84.9%, \( p < 0.001 \)). Multivariate cox analysis indicated that high Bcl-2 expression was an independent factor associated with inferior DFS (hazard ratio [HR]: 2.092; 95% confidence interval [CI]: 1.106–3.955; \( p = 0.023 \)) and OS (HR: 2.784; 95% CI: 1.326–5.846; \( p = 0.007 \)), while higher Noxa expression was associated with superior DFS (HR: 0.398; 95% CI: 0.175–0.907; \( p = 0.028 \)) and OS (HR: 0.274; 95% CI: 0.110–0.686; \( p = 0.006 \)).

Conclusions Higher expression of Bcl-2 and lower expression of Noxa were associated with unfavorable prognosis of GEP-NENs patients.

Keywords Neuroendocrine neoplasms · Bcl-2 · Apoptosis · Noxa · prognosis

Introduction

Neuroendocrine neoplasms (NENs) are a rare group of tumors with high heterogeneity, originating from neuroendocrine cells or peptide-energetic neurons. In a series of 64,971 cases with NENs reported by the Surveillance,
Epidemiology, and End Results (SEER) program of the National Cancer Institute, the annual incidence rate increased from 1.09 per 100,000 in 1973 to 6.98 per 100,000 in 2012 [1]. The therapeutic effects of chemotherapy and approved target drugs are still limited. For instance, the median progression-free survival (PFS) of patients received targeted therapies such as sunitinib and everolimus in well-differentiated pancreatic neuroendocrine tumors (NETs) were 11.4 months and 11.0 months, respectively [2, 3]. In non-pancreatic neuroendocrine tumors, the median PFS of patients received everolimus and surufatinib was 11.0 months and 9.2 months, respectively [4, 5]. Thus, development of novel targeted small molecule drugs is still an arduous subject that needs to be resolved urgently.

Apoptosis, one of the important forms of cell death, is tightly regulated by the balance of proteins in the Bcl-2 family which includes pro-apoptotic proteins (e.g., Bax, Bak), anti-apoptotic proteins (such as Bcl-2, Mcl-1, Bcl-xL) and BH3-only proteins (such as Noxa, PUMA, etc.) [6, 7]. The imbalance between cell proliferation and apoptosis, or apoptosis evasion, is of great importance for tumorigenesis [8]. In some solid and hematologic tumors, Bcl-2 over-expression is associated with more malignant phenotypes and worse prognosis [9–11]. Based on this theory, several small molecules targeting Bcl-2 were designed to re-activate apoptosis which have also been developed and tested in clinical trials. Venetoclax, for instance, has been approved by the Food and Drug Administration (FDA) for the treatment of adult chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), as well as acute myeloid leukemia (AML) by combining with azacitidine or decitabine [9, 12]. Although previous studies found that pancreatic small cell neuroendocrine carcinomas (NECs) had a higher Bcl-2 expression level than pancreatic NETs [13], the expression of Bcl-2 family proteins in GEP-NENs is still less understood.

Therefore, we aimed to further study the expression of Bcl-2 family proteins, including Bcl-2, Bcl-xL, Mcl-1, Noxa, and PUMA, in patients with GEP-NENs, and investigate their relationships with the prognosis of patients.

**Methods**

**Patient cohort and data collection**

Paraffin-embedded tumor tissue samples were collected from 105 patients who were diagnosed with GEP-NENs at The First Affiliated Hospital, Sun Yat-sen University from January 2008 to November 2016. All patients included were pathologically reassessed according to the 2019 world health organization(WHO) criteria [14] by a pathologist with extensive experience, and TNM staging was re-performed according to the eighth edition AJCC guidelines [15]. All the clinicopathological data, including gender, age, tumor grade, tumor size, lymphatic and distant metastasis status, and follow-up data, were retrospectively collected from the medical record system of The First Affiliated Hospital, Sun Yat-sen University. This study was approved by the clinical ethics committee of The First Affiliated Hospital, Sun Yat-sen University (2020489) and complied with the ethical standards of the World Medical Association Declaration of Helsinki.

**Immunohistochemical staining**

Immunohistochemistry (IHC) was carried out as followed. In brief, first, the paraffin-embedded samples were sectioned into 4μm-thick sections and dewaxed in xylene, rehydrated in rinsed graded ethanol solutions. Endogenous peroxidase activity was then blocked using 3% hydrogen peroxide solution for 10 min, and rinsed in phosphate-buffered saline (PBS) 3 times for 5 min each. Then the tissue sections were heated at 100 °C for 5 min in citrate (10 mmol/L, pH 6.0) solution to retrieve the antigens. After cooling to room temperature, serum blocker was added to block non-specific antigen, and the sections were incubated with the primary antibody Bcl-2 (ab182858; Abcam, Cambridge, MA, USA, 1:1000 dilution), Bcl-xL (cat#2764, Cell Signaling Technology, USA, 1:3000 dilution), Mcl-1 (ab32087; Abcam, Cambridge, MA, USA, 1:200 dilution), Noxa (ab13654; Abcam, Cambridge, MA, USA, 1:2000 dilution), PUMA (ab33906; Abcam, Cambridge, MA, USA, 1:200 dilution) at 4 °C overnight, followed by washing with PBS for three times, and then biotinylated goat anti-mouse IgG (1:200, Dako, Glostrup, Denmark) and all of the slides were counterstained with hematoxylin.

**Immunohistochemical analysis**

The slides were evaluated independently by two observers blinded to clinicopathological information. Any disagreement was resolved by a joint reevaluation. The expression of markers was evaluated by combining the percentage of positive cells and staining intensity. The percentage of positive cells was evaluated quantitatively and scored as 0 for staining of <2% of total cells counted, 1 for staining of 2–25%, 2 for staining of 26–50%, 3 for staining of 51–75%, and 4 for staining of > 75% of the cells examined. The intensity was graded as follows: 0, negative staining; 1, weak; 2, moderate; and 3, strong staining. A total "staining
Follow-up

All patients were followed up every three to six months in out-patient clinic or by telephone, with the last telephone follow-up in June 2020. The time of death was recorded for patients who died, and the last follow-up date and status of patients who could not be reached were obtained from the hospital system. The primary outcome was overall survival (OS), defined as the time from the date of diagnosis to death or the last follow-up. The secondary outcome of the study was disease-free survival (DFS), defined as the time from the date of diagnosis to disease recurrence or death or the last follow-up.

Statistical analyses

Statistical analyses were performed using IBM SPSS software, version 25.0 (IBM, Chicago, IL, USA). The cut-off value of markers expression was determined by the median IHC score as previous study [18, 19]. The IHC score which was greater than cut-off value was defined as high expression, while lower than cut-off value was defined as low expression. Quantitative variables were presented as mean ± average and categorical variables were presented as percentages. Chi-square test (or Fisher’s exact test) was applied to compare categorical variables. Survival analyses were performed via Kaplan–Meier method with long-rank. Univariate and multivariate analyses were carried out based on cox proportional hazard regression. Results were presented as hazard ratio (HR) and 95% confidence intervals (CI). Two sides p-value < 0.05 was considered statistically significant.

Results

Clinicopathological characteristics of patients

In total, 105 patients with GEP-NENs were included. Twenty-eight cases (26.7%) had primary tumors in the stomach, while 39 cases (37.1%) had tumors in the intestine, and 38 cases (36.2%) had tumors in the pancreas (Table 1). Sixty-three patients (60.0%) were male with an average age of 51.0 years old. As for tumor grade, grade 1, 2, 3 and NEC were found in 33 (31.4%), 27 (25.7%), 13 (12.4%), and 32 (30.5%) patients, respectively. Among these patients, 51 patients (48.6%) had lymph node metastases while 49 patients (46.7%) had distant metastases. In terms of treatment, a total of 81 patients underwent surgical resection. Twenty-four patients did not undergo surgery, among which 14 patients underwent systemic chemotherapy, including 4 with capecitabine and temozolomide (Captem), 9 with etoposide and platinum (EP), and 1 with capecitabine and oxaliplatin (XELOX). Eight patients were treated with somatostatin analogs (SSA), and the remaining two were treated with Sunitinib and Everolimus, respectively.

Table 1: Clinical data of 105 patients of GEP-NEN

| Characteristic                     | Total (n = 105) |
|-----------------------------------|-----------------|
| Sex, n (%)                        | Male 63 (60.0)  |
|                                   | Female 42 (40.0) |
| Age (x ± S) ≥60                    | 81 (77.1)       |
| Age (x ± S) >60                    | 24 (22.9)       |
| Primary tumor location, n (%)      | Stomach 28 (26.7)|
|                                   | NET 12 (11.4)   |
|                                   | Type I 4 (3.8)  |
|                                   | Type II 1 (1.0) |
|                                   | Type III 7 (6.7) |
|                                   | NEC 16 (15.2)   |
|                                   | Intestine 39 (37.1) |
|                                   | Duodenal 8 (7.6) |
|                                   | Colic 3 (2.9)   |
|                                   | Rectal 28 (26.7) |
|                                   | Pancreas 38 (36.2) |
|                                   | NET 35 (33.3)   |
|                                   | NEC 3 (2.9)     |
| Grade, n (%)                      | G1 33 (31.4)    |
|                                   | G2 27 (25.7)    |
|                                   | G3 13 (12.4)    |
|                                   | NEC 32 (30.5)   |
| T stage                           | 1 29 (27.6)     |
|                                   | 2 21 (20.0)     |
|                                   | 3 30 (28.6)     |
|                                   | 4 25 (23.8)     |
| N stage                           | 0 54 (51.4)     |
|                                   | 1 45 (42.9)     |
|                                   | 2 6 (5.7)       |
| M stage                           | 0 56 (53.3)     |
|                                   | 1 49 (46.7)     |
| Surgical operation                | Yes 24 (22.9)   |
|                                   | No 81 (77.1)    |

Score” of 0–12 was calculated by multiplying staining intensity score and staining percentage score [16, 17].

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Correlation analysis between Bcl-2 family markers and clinicopathological characteristics

As shown in Fig. 1, immunohistochemical staining for Bcl-2 and Noxa were found in cytoplasm of tumor cells. The result of Chi-square test analysis showed that there was no correlation between the expression of Bcl-2 and Noxa \((p = 0.161)\). In order to determine the relationship between the expression of Bcl-2 family markers and clinicopathological parameters, we then took the Bcl-2 family markers expressions and clinical characteristics into Chi-square test analysis (Table 2). High expression of Bcl-2 was mostly found in NEC patients (65.6% vs. 38.4%, \(p = 0.011\)), while Bcl-2 expression level showed no significant correlation with other clinicopathologic features \((p > 0.05)\). However, a diametrically opposite result was witnessed in Noxa. High expression of Noxa mostly occurred in NET patients (49.3% vs. 25.0%, \(p = 0.005)\). In addition, high Noxa expression was associated with younger age at diagnosed (48.1% vs. 20.8%, \(p = 0.017\)). Expressions of Mcl-1, Bcl-xL, and PUMA were not significantly correlated with clinicopathological characteristics (Supplementary Table 1).

Bcl-2 and Noxa are potential predictors for survival of patients with GEP-NENs

The median follow-up duration was 45 months (range 1–129 months). Forty-three patients (41.0%) died due to
tumor progression, of whom 3 cases (7.0%) having G1, 6 cases (14.0%) having G2, 8 cases (18.6%) having G3, and 26 (60.5%) cases having NEC.

Survival analysis showed that the 5-year DFS rate of patients was significantly different between the Bcl-2 high and low expression groups (34.9% vs. 68.2%, \( p < 0.001 \), Fig. 2A). Next, we divided the whole cohort into NETs and NEC subgroups for further analysis, and the results showed that the 5-year DFS rates of high and low Bcl-2 expression were significantly different only in the NET group (57.1% vs. 78.8%, \( p = 0.032 \), Fig. 2B), same trend was observed in the NEC group, but not statistically significant (9.5% vs. 24.2%, \( p = 0.090 \), Fig. 2C). Similarly, the 5-year DFS rate of Noxa high expression was significantly higher than that of Noxa low expression in the NET group (88.5% vs. 51.9%, \( p = 0.001 \), Fig. 2E), and NEC group (37.5% vs. 8.3%, \( p = 0.071 \), Fig. 2F), although not statistically significant. We also assessed the prognostic value of Bcl-2 and Noxa for OS in all cases. Kaplan–Meier survival analysis showed that both in the NET group and in the NEC group, patients in the low Bcl-2 expression group and high Noxa group had superior 5-year OS (Fig. 3). The expressions of Bcl-xl, Mcl-1, and PUMA were not correlated with DFS (S-Fig. 1) or OS (S-Fig. 2).

As shown in Table 3, univariate and multivariate Cox regression analyses were used to identify risk factors for NENs patients. Univariate Cox regression analyses demonstrate that age above 60 years old (\( p < 0.001 \)), Grade 3 (\( p < 0.001 \)), NEC (\( p < 0.001 \)), T4 stage (\( p = 0.012 \)), N1-2 stage (\( p = 0.041 \)), M1 stage (\( p = 0.005 \)), and high Bcl-2 expression (\( p < 0.001 \)) were correlated with higher risk of disease recurrence, while pancreas NENs (\( p = 0.005 \)), operation (\( p < 0.001 \)), and high Noxa expression (\( p < 0.001 \)) were correlated with lower risk of disease recurrence. Multivariate cox regression analyses indicated that Grade 3 (\( p = 0.006 \)), NEC (\( p < 0.001 \)), N1-2 stage (\( p = 0.015 \)), M1 stage (\( p = 0.024 \)), operation (\( p = 0.012 \)), Bcl-2 expression (\( p = 0.023 \)), and Noxa expression (\( p = 0.028 \)) were independent parameters of disease recurrence. As for OS, the univariate analysis and multivariate cox analysis indicated that NEC (\( p < 0.001 \)), operation (\( p = 0.007 \)), the Bcl-2 levels (\( p = 0.007 \)), and Noxa expression (\( p = 0.006 \)) were independent parameters of higher risk of death.

| Parameters          | Expression of Bcl-2 | p value* | Expression of Noxa | p value* |
|---------------------|--------------------|----------|-------------------|---------|
|                     | Low (n = 56, %)    | High (n = 49, %) |                   | Low (n = 61, %) | High (n = 44, %) |
| Age, year           |                    |          |                   |         |
| ≤60                 | 45 (55.6)          | 36 (44.4) | 0.402             | 42 (51.9) | 39 (48.1)       | 0.017 |
| >60                 | 11 (45.8)          | 13 (54.2) | 0.873             | 19 (79.2) | 5 (20.8)        | 0.170 |
| Sex                 |                    |          |                   |         |
| Male                | 34 (54.9)          | 29 (46.0) | 0.873             | 40 (63.5) | 23 (36.5)       | 0.024 |
| Female              | 22 (52.4)          | 20 (47.6) | 0.210             | 21 (50.0) | 21 (50.0)       |       |
| Primary tumor location |                |          |                   |         |
| Stomache            | 12 (42.9)          | 16 (57.1) | 0.400             | 21 (75.0) | 7 (25.0)        | 0.024 |
| Intestine           | 23 (59.0)          | 16 (41.0) | 0.400             | 24 (61.5) | 15 (38.5)       |       |
| Pancreas            | 21 (55.3)          | 17 (44.7) | 0.400             | 16 (42.1) | 22 (57.9)       |       |
| WHO Grade           |                    |          |                   |         |
| G1                  | 24 (72.7)          | 9 (27.3)  | 0.011             | 14 (42.4) | 19 (57.6)       | 0.005 |
| G2                  | 16 (59.3)          | 11 (40.7) | 0.126             | 12 (44.4) | 15 (55.6)       |       |
| G3                  | 5 (38.5)           | 8 (61.5)  | 0.024             | 11 (64.4) | 2 (15.4)        |       |
| NEC                 | 11 (34.4)          | 21 (65.6) | 0.024             | 24 (75.0) | 8 (25.0)        |       |
| T stage             |                    |          |                   |         |
| 1–3                 | 46 (57.5)          | 34 (42.5) | 0.126             | 44 (55.0) | 36 (45.0)       | 0.250 |
| 4                   | 10 (40.0)          | 15 (60.0) | 0.126             | 17 (68.0) | 8 (32.0)        |       |
| N stage             |                    |          |                   |         |
| 0                   | 32 (59.3)          | 22 (40.7) | 0.210             | 27 (50.0) | 27 (50.0)       | 0.084 |
| 1–2                 | 24 (47.1)          | 27 (52.9) | 0.210             | 34 (66.7) | 17 (33.3)       |       |
| M stage             |                    |          |                   |         |
| 0                   | 32 (57.1)          | 24 (42.9) | 0.403             | 30 (53.6) | 26 (46.4)       | 0.315 |
| 1                   | 24 (49.0)          | 25 (51.0) | 0.403             | 31 (63.3) | 18 (36.7)       |       |

* \( X^2 \) test, a, Pearson’s Chi-Square test

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Table 2 relationship between the expression of Bcl-2, Noxa and clinicopathologic parameters
Discussion

In order to further explore prognostic biomarkers for long-term survival in GEP-NENs, our study evaluated the Bcl-2 family protein expressions in tumor tissue of GEP-NENs patients. Our results indicated that Bcl-2 and Noxa expressions correlate with tumor grade and survival prognosis. Overexpression of Bcl-2 protein and low expression of Noxa protein indicated poor tumor differentiation and poor prognosis.

Bcl-2 is the fundamental member of Bcl-2 family of apoptosis and is classified as an oncogene [20]. Dysregulation of Bcl-2 family proteins has been found in a variety of tumors such as lung cancer, melanoma, and AML [21–23]. In this study, we found that the expression of Bcl-2 in NEC was significantly higher than that in NET, and the same trend was also observed in previous studies which focused on pancreatic NENs, suggesting that overexpression of Bcl-2 may be responsible for higher proliferation rate and more malignant phenotype of NENs [13]. This phenomenon was also observed in NENs derived from lung. The expression of Bcl-2 in small cell lung cancer (SCLC) was higher than that in typical carcinoid (TC) and atypical carcinoid (AC). What’s more, several studies found a positive correlation between Bcl-2 and chromogranin A (CgA), which indicated the expression of Bcl-2 may be involved in neuroendocrine differentiation [13, 21, 24]. In addition, overexpression of Bcl-2 also induces resistance to chemotherapy and targeted therapies [25, 26]. Currently, several Bcl-2 inhibitors, including Venetoclax, have...
demonstrated marked activity in vitro studies and good pharmacological effects in clinical trials and have been approved by FDA for the treatment of SCLC, AML, and CLL [27–29]. We found overexpression of Bcl-2 in poorly differentiated GEP-NEC, suggesting that inhibitors targeting Bcl-2 may also be a potential treatment option for these patients.

On the contrary, Noxa, which belongs to a subclass of BH3-only proteins, selectively binds to Mcl-1 and plays a pro-apoptotic effect through the neutralization of Mcl-1 [30, 31]. Previous studies have revealed that Noxa gene expression and protein function have been linked to cell death in kinds of hematopoietic and solid cancers, such as melanoma, multiple myeloma (MM), and CLL [31–33]. In melanoma and breast cancer studies, induced upregulation of Noxa enhanced the pharmacological effects of BH3 analogue ABT-737 [34, 35]. In our study, we also observed that the expression of Noxa was significantly higher in NET, compared with NEC, and was positively associated with prognosis. Restoring or enhancing Noxa expression may significantly increase treatment efficacy and may serve as a worthwhile strategy to be explored in GEP-NENs.

This study still has some limitations. First of all, this is a retrospective study, with inherent limitations of retrospective research, such as missing some data. Secondly, given the limitations of the understanding of NENs in the early years, some patients did not receive standard treatment. Therefore, further large sample and multi-center studies are of great importance to validate these conclusions.
Table 3 Univariate and multivariate Cox regression analysis of overall survival and disease-free survival

| Variable                              | Disease-free Survival | Overall Survival |
|---------------------------------------|-----------------------|------------------|
|                                       | Univariate            | Multivariate     | Univariate | Multivariate |
|                                       | HR (95%CI) p          | HR (95%CI) p     | HR (95%CI) | HR (95%CI) p |
| Age (≤60 year vs. >60 year)           | 3.269 (1.816–5.883) <0.001 | 1.125 (0.488–2.591) 0.783 | 3.631 (1.947–6.772) <0.001 | 1.151 (0.478–2.772) 0.753 |
| Sex (Male vs. Female)                 | 0.882 (0.498–1.562) 0.667 |                  | 0.844 (0.455–1.567) 0.591 |                  |
| Primary tumor location (stomach vs. intestine) | 0.602 (0.316–1.149) 0.124 | 1.667 (0.826–3.366) 0.154 | 2.627 (1.248–5.531) 0.011 | 1.425 (0.665–3.051) 0.362 |
| Primary tumor location (stomach vs. pancreas) | 0.364 (0.179–0.741) 0.005 | 1.342 (0.492–3.663) 0.566 | 1.380 (0.637–2.989) 0.663 | 1.992 (0.704–5.635) 0.194 |
| WHO Grade                             |                       |                  |             |             |
| G2 vs. G1                             | 3.683 (0.996–13.617) 0.051 | 2.946 (0.736–11.792) 0.127 | 2.451 (0.612–9.813) 0.205 | 1.259 (0.289–5.494) 0.759 |
| G3 vs. G1                             | 14.509 (3.956–53.208) <0.001 | 8.228 (1.852–36.550) 0.006 | 9.042 (2.321–35.222) 0.002 | 1.978 (0.404–9.683) 0.400 |
| NEC vs. G1                            | 21.734 (6.537–72.256) <0.001 | 28.084 (6.882–114.60) <0.001 | 18.376 (5.502–61.366) <0.001 | 14.999 (3.796–59.261) <0.001 |
| T (1-3 vs. 4)                         | 2.158 (1.187–3.921) 0.012 | 1.058 (0.519–2.159) 0.876 | 1.895 (0.992–3.619) 0.053 |                  |
| N (0 vs.1-2)                          | 1.806 (1.025–3.183) 0.041 | 0.439 (0.226–0.853) 0.015 | 1.646 (0.898–3.018) 0.107 |                  |
| M (0 vs. 1)                           | 2.278 (1.283–4.043) 0.005 | 2.264 (1.113–4.605) 0.024 | 2.023 (1.095–3.736) 0.024 | 1.689 (0.832–3.427) 0.147 |
| Operation (No vs. Yes)                | 0.326 (0.183–0.581) <0.001 | 0.369 (0.170–0.803) 0.012 | 0.322 (0.174–0.597) <0.001 | 0.312 (0.134–0.726) 0.007 |
| Bcl-2 expression (low vs. high)       | 2.949 (1.643–5.295) <0.001 | 2.092 (1.106–3.955) 0.023 | 3.669 (1.914–7.032) <0.001 | 2.784 (1.326–5.846) 0.007 |
| Noxa expression (low vs. high)        | 0.293 (0.152–0.563) <0.001 | 0.398 (0.175–0.907) 0.028 | 0.216 (0.099–0.469) <0.001 | 0.274 (0.110–0.686) 0.006 |
| Bcl-xl expression (low vs. high)      | 0.860 (0.476–1.555) 0.618 |                  | 0.912 (0.483–1.723) 0.777 |                  |
| Puma expression (low vs. high)        | 0.758 (0.430–1.336) 0.338 |                  | 0.823 (0.449–1.511) 0.530 |                  |
| Mcl-1 expression (low vs. high)       | 0.866 (0.494–1.519) 0.615 |                  | 0.720 (0.388–1.337) 0.299 |                  |

*HR* hazard ratio, 95% CI, 95% confidence interval
Conclusion

Taken together, our study, for the first time, systematically detected the expression of Bcl-2 family proteins in GEP-NENs and further evaluated the relationship between the expression of Bcl-2 family proteins and the prognosis of GEP-NENs patients. Our results demonstrated that Bcl-2 and Noxa were valuable and independent prognostic markers of DFS and OS in GEP-NENs. It gave us a new understanding of NENs and laid the ground for the application of drugs targeting Bcl-2 family proteins in treating NENs.

Author contributions All authors contributed to the study conception and design. Data collection and analysis were performed by Y.G., L.Z., and N.Z. Experiments design: D.J.Y. and J.C. Immunohistochemical staining and analysis: M.L., Q.Y.L. Manuscript writing: Y.G., L.Z., N.Z., and L.C. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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References

1. A. Dasari, C. Shen, D. Halperin, B. Zhao, S. Zhou, Y. Xu et al. Trends in the incidence, prevalence, and survival outcomes in patients with neuroendocrine tumors in the United States. JAMA Oncol. 3(10), 1335–1342 (2017)
2. J.C. Yao, M.H. Shah, T. Ito, C.L. Bohas, E.M. Wolin, E. Van Cutsem et al. Everolimus for advanced pancreatic neuroendocrine tumors. N. Engl. J. Med. 364(6), 514–523 (2011)
3. E. Raymond, L. Dahan, J.L. Raoul, Y.J. Bang, I. Borbath, C. Lombard-Bohas et al. Sunitinib malate for the treatment of pancreatic neuroendocrine tumors. N Engl J Med. 364(6), 501–513 (2011)
4. J.C. Yao, N. Fazio, S. Singh, R. Buzzoni, C. Carnaghi, E. Wolin et al. Everolimus for the treatment of advanced, non-functional neuroendocrine tumours of the lung or gastrointestinal tract (RADIANT-4): a randomised, placebo-controlled, phase 3 study. Lancet 387(10022), 968–977 (2016)
5. J. Xu, L. Shen, Z. Zhou, J. Li, C. Bai, Y. Chi et al. Surufatinib in advanced extrapancreatic neuroendocrine tumours (SANET-ep): a randomised, double-blind, placebo-controlled, phase 3 study. Lancet Oncol. 21(11), 1500–1512 (2020)
6. J.E. Guikema, M. Amiot, E. Eldering. Exploiting the proapoptotic function of NOXA as a therapeutic modality in cancer. Expert Opin. Ther. Targets 21(8), 767–779 (2017)
7. E.M. Bruckheimer, S.H. Cho, M. Sarkiss, J. Herrmann, T.J. McDonnell, The Bcl-2 gene family and apoptosis. Adv. Biochem. Eng. Biotechnol. 62, 75–105 (1998)
8. J.B. Dietrich, Apoptosis and anti-apoptosis genes in the Bcl-2 family. Arch. Physiol. Biochem. 105(2), 125–135 (1997)
9. I. Kapoor, J. Bodo, B. Hill, E. Hsi, A. Almasan. Targeting BCL-2 in B-cell malignancies and overcoming therapeutic resistance. Cell Death Dis. 11(11), 941 (2020)
10. J.W. Moul, Angiogenesis, p53, bcl-2 and Ki-67 in the progression of prostate cancer after radical prostatectomy. Eur. Urol. 35(5-6), 399–407 (1999)
11. Y. Wei, Y. Cao, R. Sun, L. Cheng, X. Xiong, X. Jin et al. Targeting Bcl-2 proteins in acute myeloid leukemia. Front. Oncol. 10, 584974 (2020)
12. N. Gangat, A. Tefferi, Venetoclax-based chemotherapy in acute and chronic myeloid neoplasms: literature survey and practice points. Blood Cancer J. 10(11), 122 (2020)
13. S. Yachida, E. Vakiani, C.M. White, Y. Zhong, T. Saunders, R. Morgan et al. Small cell and large cell neuroendocrine carcinomas of the pancreas are genetically similar and distinct from well-differentiated pancreatic neuroendocrine tumors. Am. J. Surg. Pathol. 36(2), 173–184 (2012)
14. I.D. Nagtegaal OR, D. Klimstra, WHO classification of tumours. Fifth Edition: World Health Organisation Press. 2019
15. S.B. Edge, AJCC cancer staging manual 8th ed: Springer; 2017
16. D. Creytens, NKX2.2 immunohistochemistry in the distinction of Ewing sarcoma from cytromorphologic mimics: Diagnostic utility and pitfalls-Comment on Russell-Goldman et al. Cancer Cytopathol. 127(3), 202 (2019)
17. Z. Guo, X. Zhang, H. Zhe, H. Zhe, H. Luo, Y. Zhang et al. TEL02 induced progression of colorectal cancer by binding with RICTOR through mTORC2. Oncol. Rep. 45(2), 523–534 (2021)
18. W. Chen, J. Peng, Q. Ou, Y. Wen, W. Jiang, Y. Deng et al. Expression of NDRG2 in human colorectal cancer and its association with prognosis. J. Cancer 10(15), 3373–3380 (2019)
19. J. Peng, Y. Zhao, Q. Luo, H. Chen, W. Fan, Z. Pan et al. High WNT6 expression indicates unfavorable survival outcome for patients with colorectal liver metastasis after liver resection. J. Cancer 10(12), 2619–2627 (2019)
20. A.S. Ebrahim, H. Sabbagh, A. Liddane, A. Raufi, M. Kandouz, A. Al-Katib, Hematologic malignancies: newer strategies to counter the BCL-2 protein. J. Cancer Res. Clin. Oncol. 142(9), 2013–2022 (2016)
21. D.G. Wang, C.F. Johnston, J.M. Sloan, K.D. Buchanan, Expression of Bcl-2 in lung neuroendocrine tumours: comparison with p53. J. Pathol. 184(3), 247–251 (1998)
22. M. Rahmani, J. Nkwocha, E. Hawkins, X. Pei, R.E. Parker, M. Tschida et al. NKX2.2 immunohistochemistry in the distinction of pancreatic neuroendocrine tumors. Am. J. Surg. Pathol. 34(6), 737–746 (2010)
23. D. Trisciuoglio, M. Desideri, L. Ciuffreda, M. Mottolese, D. Ribatti, A. Vacca et al. Bcl-2 overexpression in melanoma cells increases tumor progression-associated properties and in vivo tumor growth. J. Cell Physiol. 205(3), 414–421 (2005)
24. A.A. Gal, M.N. Sheppard, J.D. Nolen, M. Cohen, p53, cellular proliferation, and apoptosis-related factors in thymic neuroendocrine tumors. Mod. Pathol. 17(1), 33–39 (2004)
25. T.C. Fisher, A.E. Milner, C.D. Gregory, A.L. Jackman, G.W. Aherne, J.A. Hartley et al. bcl-2 modulation of apoptosis induced by anticancer drugs: resistance to thymidylate stress is independent of classical resistance pathways. Cancer Res. 53(14), 3321–3326 (1993)
26. U.A. Sartorius, P.H. Krammer, Upregulation of Bcl-2 is involved in the mediation of chemotherapy resistance in human small cell lung cancer cell lines. Int J. Cancer 97(5), 584–592 (2002)
27. S. Hafezi, M. Rahmani, Targeting BCL-2 in cancer: advances, challenges, and perspectives. Cancers. 2021;13
28. T.L. Lochmann, K.V. Floros, M. Naseri, K.M. Powell, W. Cook, R.J. March et al. Venetoclax Is Effective in Small-Cell Lung Cancers with High BCL-2 Expression. Clin. Cancer Res. 24(2), 360–369 (2018)

29. D.A. Pollyea, Venetoclax in AML: where we are and where we are headed. Clin. Lymphoma Myeloma Leuk. 20(Suppl 1), S25–S26 (2020)

30. P. Gomez-Bougie, S. Wuilleme-Toumi, E. Menoret, V. Trichet, N. Robillard, M. Philippe et al. Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. Cancer Res. 67(11), 5418–5424 (2007)

31. K.G. Ponder, S.M. Matulis, S. Hitosugi, V.A. Gupta, C. Sharp, F. Burrows et al. Dual inhibition of Mcl-1 by the combination of carfilzomib and TG02 in multiple myeloma. Cancer Biol. Ther. 17 (7), 769–777 (2016)

32. M.C. Albert, K. Brinkmann, H. Kashkar. Noxa and cancer therapy: Tuning up the mitochondrial death machinery in response to chemotherapy. Mol. Cell Oncol. 1(1), e29906 (2014)

33. W.J. Mackus, A.P. Kater, A. Grummels, L.M. Evers, B. Hooijbrink, M.H. Kramer et al. Chronic lymphocytic leukemia cells display p53-dependent drug-induced Puma upregulation. Leukemia 19(3), 427–434 (2005)

34. K.M. Lucas, N. Mohana-Kumaran, D. Lau, X.D. Zhang, P. Hersey, D.C. Huang et al. Modulation of NOXA and MCL-1 as a strategy for sensitizing melanoma cells to the BH3-mimetic ABT-737. Clin. Cancer Res. 18(3), 783–795 (2012)

35. C. Seveno, D. Loussouarn, S. Brechet, M. Campone, P. Juin, S. Barille-Nion, gamma-Secretase inhibition promotes cell death, Noxa upregulation, and sensitization to BH3 mimetic ABT-737 in human breast cancer cells. Breast Cancer Res. 14(3), R96 (2012)