Prognostic Potential of BCL-2 and FOXP3 Expression in Bladder Carcinoma

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Abstract:

Background: bladder cancer develops and spreads due to a combination of environmental and inherited causes. Many biomarkers that are useful in determining the behavior and prognostic features of the tumour have been studied. Bcl-2 overexpression was linked to reduced tumour sensitivity to chemo- and radiation. FOXP3 has multiple roles in tumoural–immune system interactions, debates over FOXP3's function in immunological responses and carcinogenesis at various sites have emerged. Aim of the study: to detect Bcl-2 & tumoral FOXP3 immunohistochemical expression in urothelial carcinoma and its correlation with other clinicopathological data as well as disease free survival (DFS) and overall survival (OS).

Methods: sixty cases of urothelial carcinoma had full clinical and follow-up data. Bcl-2 & tumoral FOXP3 immunohistochemical expression in urothelial carcinoma cases were investigated.

Results: Positive Bcl-2 expression was detected in 41.7% of the studied urothelial carcinoma cases, while two thirds of cases showed positive FOXP3 expression. A statistically significant association between loss of Bcl-2 expression and high grade, advanced T stage, the presence of lymph node metastasis and lympho-vascular invasion (p=0.000) was detected. A statistically significant association between positive FOXP3 expression and both positive lymph node metastasis and lympho-vascular invasion was detected (p= 0.02 and p= 0.007 respectively). No significant association between Bcl-2 expression and DFS as well as OS, meanwhile, a statistically significant association between positive FOXP3 expression and short DFS as well as OS.

Conclusion: The identification of patients with poor prognostic characteristics in urothelial bladder cancer who may be candidates for cancer therapy could be facilitated by loss of Bcl-2 expression and positive tumoral FOXP3 expression.

Keywords: Bladder cancer, Immunohistochemistry, Bcl-2, Tumoral FOXP3.

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Introduction:

Bladder cancer is the most common malignant tumour of the urinary tract and the ninth most common cancer worldwide. More White people than African people and more men than women are affected by this malignancy. More than 90% of instances of primary bladder cancer are attributed to urothelial carcinoma. [1].

More common types of bladder cancer include adenocarcinomas and squamous cell carcinoma. Like most cancers, bladder cancer develops and spreads due to a combination of environmental and genetic causes. [2]. Bladder tumour frequency is higher in industrial areas and increases with exposure to arylamines and smoking, primarily due to chemical factors. The disease's most typical symptom is either microscopic or gross hematuria, which is followed by secondary urinary tract infection (UTI) symptoms [3]. Transurethral resection is the surgical procedure used to treat bladder cancer. This procedure is most effective in treating well-differentiated, non-invasive bladder tumours (pTa and pT1), which account for 75% of bladder cancer cases. However, following resection, a recurrence is expected in up to 70% of individuals with superficial urothelial carcinoma, and 10% to 15% will progress to invasive tumours. [4]. About 70% to 80% of bladder cancers were superficial tumors when they were first diagnosed, and the majority of them (about 70%)

are prone to recur. Additionally, 15% of bladder cancers may proceed to become muscle-invasive diseases even in the presence of adjuvant immune treatment or chemotherapy.[5].

Despite the lack of a uniform biomarker for clinical outcome prediction, a number of conventional tumor markers and molecular pathways associated with these tumors or treatment outcomes have been studied. For instance, it has been revealed that p53, vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) are crucial for the development of bladder cancer and tumor recurrence [6, 7].

Bladder cancer develops as a result of several changes in the activity of genes that regulate cell division and death. Apoptosis is regulated by the B-cell lymphoma 2 (Bcl-2) protein family and a number of additional genes, including c-myc, H ras, ABL, Apo-1, and p53 [8]. Bcl-2 overexpression is proved to be linked to reduced tumour sensitivity to chemo- and radiation. In light of this, it was postulated that pathogenesis, development, and responsiveness to therapeutic interventions of bladder cancer are all greatly influenced by Bcl-2 overexpression [8].

Forkhead box P3 (Foxp3) is an X-linked transcription factor that is required for induction of the immunosuppressive functions in regulatory T lymphocytes. Although its expression was first considered to be specific to this cell type, various studies have demonstrated that Foxp3 protein is expressed as a marker for regulatory T cells (Treg) cells and as an onco-suppressor in several mice models, including breast, prostate, and pancreatic cancers via either as a transcriptional repressor of c-Myc, Skp2, and HER2 gene expression, or a regulator of interleukin (IL)−6 or −8 expression [9-11].

Numerous investigations have demonstrated that cancer cells are also capable of producing FOXP3. This expression is associated with levels of transforming growth factor-β and interleukin-10, and co-culturing naïve T-cells with FOXP3-expressing cancer cells from a pancreatic carcinoma entirely reduced proliferation. FOXP3 expression in cancers is not restricted to any one kind of tumour; it can be found in both mesenchymal and epithelial tumours as well as hematological malignancies.[12]. Tumor-FOXP3 plays contradictory, sometimes even opposite, functions. In breast cancer (BC) [13], gastric cancer (GC) [14] and Hepatocellular carcinoma (HCC) [15], tumour FOXP3 functions as a suppressor of tumours, preventing the production of several oncogenes. On the other hand, in pancreatic cancer, tumour FOXP3 has been found to be a biomarker linked to a malignant prognosis. In contrast, a biomarker linked to a malignant prognosis in pancreatic cancer [16], Non-small cell lung cancer (NSCLC) [17] and thyroid cancer [18] has been discovered as tumour FOXP3. A study on breast cancer patients who had tumoral expression of FOXP3 showed a negative association with survival. Thus, it seems that FOXP3 plays a variety of roles in the interactions between the immune system and tumours. [9]. As a result, debates over FOXP3's function in immunological responses and carcinogenesis at various sites have emerged.

Our study aims to detect Bcl-2 & FOXP3 immunohistochemical expression in urothelial carcinoma and its correlation with other clinicopathological data as well as disease free survival (DFS) and overall survival (OS).

Patients and Methods:

Patient selection criteria:

This retrospective analysis included 60 cases of urothelial carcinoma that were treated at the South Egypt Cancer Institute in 2019–2020. The formalin fixed paraffin embedded tissues were obtained from the Oncological pathology Department, South Egypt Cancer Institute, Assiut University. All H&E slides were re-examined to confirm diagnosis of urothelial carcinoma. Grading and Pathological staging according to WHO 2016 [19]. Cases received neoadjuvant chemotherapy; cases presented with metastasis at time of diagnosis as well as cases with recurrent urothelial carcinoma were excluded. Records belonging to the patients were used to gather data on survival and clinicopathological characteristics. The study was authorized by Institutional Review Board (IBR) - Ethical approval No. 664.

Immunohistochemical staining:

Tissue that had been embedded in paraffin and fixed with formalin was cut into 5-micron sections and mounted on two slides that were positively charged. After tissues were deparaffinized, they were rehydrated using distilled water and graded alcohols. Antigen retrieval was carried out by incubating slides in Tris EDTA in a hot water bath set at 90 degrees for 45 minutes. Hydrogen peroxide block was applied and incubated for 10-15 minutes at room temperature to reduce endogenous peroxidase activity. Then ultra V block was applied to the slides and incubated 5 minutes at room temperature in humidity chamber to prevent unnecessary back ground staining. Primary mouse monoclonal anti- Human Bcl-2, antibody (M0887, DAKO, CA, USA) at 1/100 concentration (optimum dilution) was applied for tissue sections and incubated for one hour at room temperature in a humid chamber. Primary rabbit monoclonal anti- forkhead-box protein P3 (FOXP3) antibody (catalog bsm-52079RUSBiological life science, USA) at 1/200, overnight at 4 c. After washing, immunostaining was performed using a universal staining kit “UltraVision Detection System Anti-Polyvalent, HRP/DAB (Ready-To-Use)” (LAB VISION corporation, catalogue # TP-015-HD, Fremont, California 94539-6406, USA) following the manufacturer’s instructions. Finally, (DAB solution) was applied to the slides for 5- 10 minutes. Mayer’s hematoxylin, was used as a counter stain. Reactive lymph node sections and human tonsil sections were utilized as positive controls for Bcl-2 and FOXP3 antibodies, respectively. As a negative control, we employed tissue-specific positive control sections as

a negative control and stained them according to the same process, excluding the primary antibody.

Interpretation of Immunohistochemical staining:

Bcl-2 & FOXP3 were detected in the cytoplasm of tumor cells (Fig.1). Special pathologists blinded to the clinicopathological data analyzed the immune-stained slides to assess its expression. When immunohistochemical staining showed positive results in less than 10% of tumour cells and the staining intensity was low, Bcl-2 and FOXP3 were considered negative. Bcl-2 and FOXP3 were regarded as positive in all other cases. [20, 21].

Survival analysis:

Survival data of the patients were obtained by reviewing the files of patients attending to South Egypt Cancer institute in the time period between 2019-2023.

Statistical analysis:

Data were gathered. Version 26 of SPSS (Statistical package for the social science; SPSS Inc., Chicago, IL, USA) was used for tabulation and statistical analysis. When not normally distributed, quantitative data were statistically characterized using mean ± SD, median, and range. When appropriate, statistical descriptions of qualitative data were given in terms of frequencies (number of cases) and relative frequencies (percentages). The student t test for normally distributed data and the Mann Whitney U test for non-normally distributed data were used to compare the quantitative variables. The Chi square ($\chi 2$) test was used to compare categorical data. Instead, when the expected frequency is less than five, the Fisher Exact test was utilized. Significant factors related with mortality were identified using COX regression analysis and the Hazard Ratio (HR) with 95% Confidence Interval (CI). P-value is always 2 tailed set significant at 0.05 level.

Results:

In this study, our results revealed that 41.7% (n=25) of the studied urothelial carcinoma cases showed Bcl-2 positive cytoplasmic expression, whereas 58.3% (n=35) of the studied cases showed negative expression. For FOXP3 expression, two thirds of cases showed positive cytoplasmic FOXP3 expression as shown in Figure 1 (A-F) & (Table 1). As regard to Bcl-2 expression, statistically significant association was detected between loss of Bcl-2 expression and high grade, advanced T stage, presence of lymph node metastasis and lympho-vascular invasion (LVI) (P=0.000) (Table 2). However, there is no significant association was detected between Bcl-2 expression and DFS as well as OS (Table 4).

Concerning FOXP3, statistically significant association was demonstrated between positive expression of FOXP3 and positive lymph node metastasis, LVI and higher grade (P= 0.02, 0.007 and 0.044 respectively). Also, statistically significant association between positive tumoral FOXP3 and both short DFS and advanced short OS was also demonstrated. Furthermore, our findings demonstrated no significant association between Bcl-2 expression and FOXP3 expression in tumor cells was detected (Table 3).

Table (1): Clinicopathologic characteristics of studied urothelial carcinoma cases (60)

Clinicopathologic characteristics	Number of cases	**P**ercent
SEX		
Male	47	78.3
female	13	21.7
Grade		
Low	31	51.7
High	29	48.3
T Stage		
Ta	31	51.7
T2	8	13.3
T3	19	31.7
T4	2	3.3
N Stage		
N0	44	73.3
N1	5	8.3
N2	11	18.3
LVI		
Yes	16	26.7
No	44	73.3
Bcl-2		
Positive	25	41.7
Negative	35	58.3
FOXP3		
Positive	40	66.6
Negative	20	33.3
Recurrence		
Yes	57	95
No	3	5
Death		
Died	59	98.3
Alive	1	1.7
Age	64	
Size	4±1	

LVI: lympho-vascular invasion

Table (2): Association between Bcl-2 expression and clinicopathologic features.

Clinicopathologic features	Bcl-2 Expression		P value
	Positive	Negative	
Sex			0.758
Male	19	28	
Female	6	7	
Grade			0.000*
Low	25	6	
High	0	29	
T stage			0.000*
Ta	25	6	
T2	0	8	
T3	0	19	
T4	0	2	
N stage			0.000*
N0	25	19	
N1	0	5	
N2	0	11	
LVI			0.000*
Yes	0	16	
No	25	19	

LVI: lympho-vascular invasion
* significant association

Table (3): Association between tumoral FOXP3 expression and clinicopathologic features.

Clinicopathologic features	FOXP3 Expression		P value
	Positive	Negative	
Sex			0.375
Male	30	17	
Female	10	3	
Grade			0.044*
Low	17	14	
High	23	6	
T stage			0.213
Ta	17	14	
T2	6	2	
T3	15	4	
T4	2	0	
N stage			0.02*
N0	25	19	
N1	4	1	
N2	11	0	
LVI			0.007*
Yes	15	1	
No	25	19	
Bcl-2			0.139
Negative	26	9	
Positive	14	11	
Age			
<64	21	9	0.584
≥64	19	11	

LVI: lympho-vascular invasion
* significant association

Table (4): Disease-free survival and Overall survival and according to clinicopathologic features of the studied urothelial carcinoma cases.

Clinicopathologic features	DFS (3 years)		OS (3 years)	
	Estimate ± SE	P value	Estimate ± SE	P value
Sex				
Male	15 ± 2.7	0.594	20 ± .833	0.978
Female	14 ± 1.7		17 ± 1.7	
Grade				
Low	12±1.3	0.516	18± 2.2	0.473
High	16±1.5		20± 1.3	
T stage				
Ta	12±1.3		18± 2.21	
T2	18± 6.3	0.178	26 ± 4.7	0.047*
T3	16 ± 1.7		20 ± 1.4	
T4	7.000		11.0 ± 0.0	
N stage				
N0	15 ± 2.4		20 ± 1.1	
N1	12.1.09	0.046*	18± 3.2	0.034*
N2	12 ± 2.4		16 ± .739	
LVI				
Yes	12 ± .992	0.016*	16 ± .645	0.011*
No	15 ± 2.4%		20 ± 1.1	
Bcl-2				
Positive	14 ± 4.04	0.773	18 ± 3.3	0.919
Negative	15 ± 1.4		20 ± 1.07	
FOXP3				
Positive	20±1.6	.000*	20±1.5	.000*
Negative	40±0.9015		40±1.016	

DFS: disease-free survival
OS: Overall survival
LVI: lympho-vascular invasion
* significant association

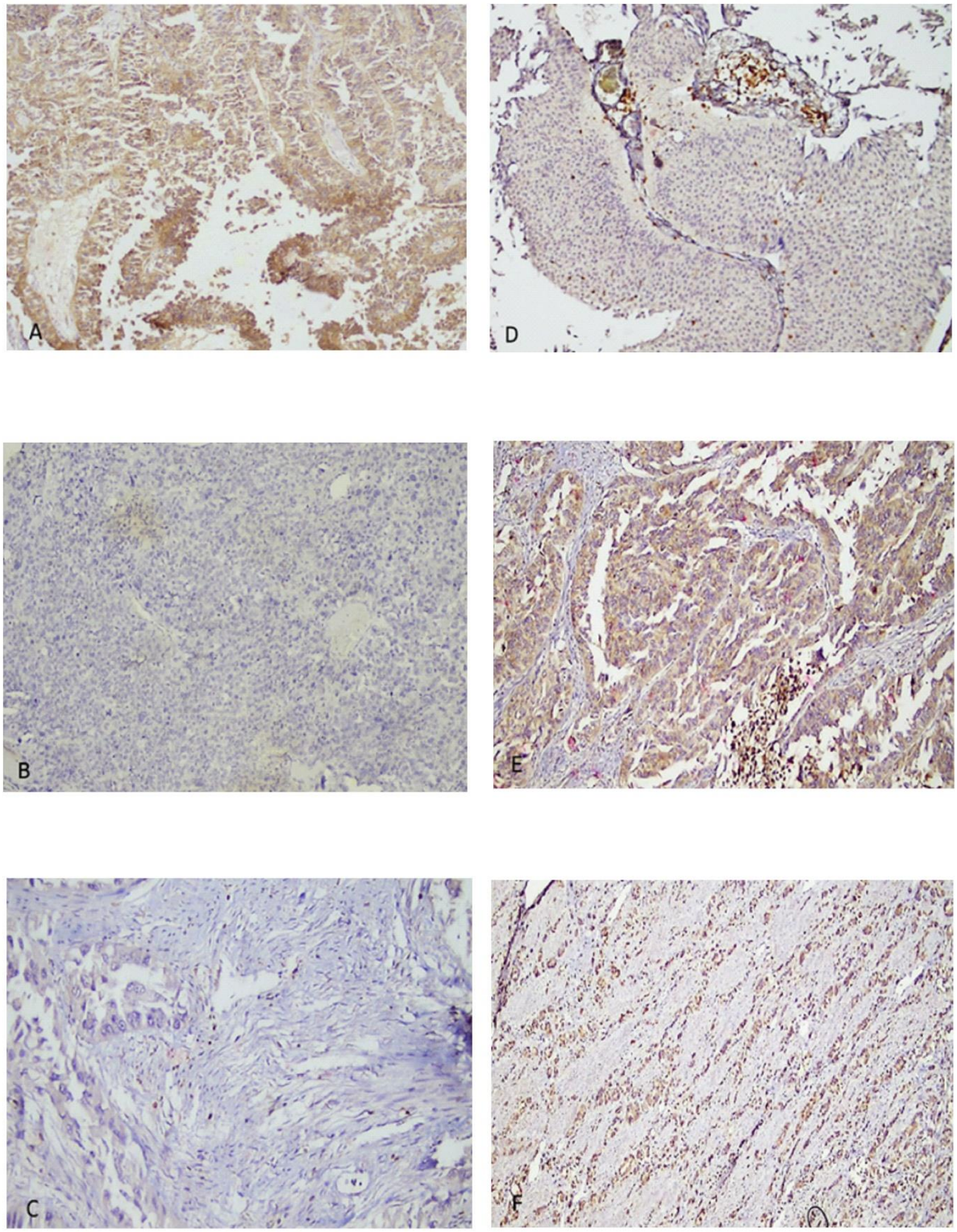

Figure 1. Different Immunohistochemical staining patterns of Bcl-2 and FOXP3 among urothelial urinary bladder cancer cases. (A) Bcl-2 positive cytoplasmic expression in low grade non-invasive papillary urothelial carcinoma X100. (B) Bcl-2 negative expression in high grade invasive urothelial carcinoma X100. (C) Bcl-2 negative expression in high grade invasive urothelial carcinoma X200. (D) FOXP3 negative expression in low grade non-invasive papillary urothelial carcinoma X100. (E) FOXP3 positive cytoplasmic expression in high grade invasive papillary X100. (F) FOXP3 positive cytoplasmic expression in high grade invasive urothelial carcinoma urothelial X100.

Discussion:

Together with pathological features, biomarkers are commonly used to assess cancer behaviors such as tumour grade and stage, neurological and vascular invasions, distant metastases, and responsiveness to therapy measures. In order to evaluate the biological characteristics of cancer and differentiate it from benign tissues, special emphasis has been made on the evaluation of highly specific and sensitive biomarkers [2]. Numerous research evaluating biomarkers for bladder cancer have found that some biomarkers are helpful in predicting the tumor's behavior and prognostic characteristics. It is still up for debate, nevertheless, which of these markers can accurately predict the biological behavior of cancer or perhaps take the place of the most accurate pathology tools. The expression of Bcl-2 in various malignancies depends on the cell line. Bcl-2 positive patients have a better prognosis than negative patients with lung and breast cancer, however Bcl-2 overexpression is a poor prognostic factor for high-grade lymphoma, leukaemia, neuroblastoma, and prostate cancer. Tissue specificity, tumour heterogeneity, or the bcl-2 expression assessment technique could all be contributing factors to these variations. [22]

In the present study, a significant association between loss of Bcl-2 expression and bad prognostic factors such as high grade, more depth of invasion, presence of LVI & L.N metastasis was evident. This was in accordance with Pourebrahimi et al. 2022 who demonstrated that lower expression of Bcl-2 was associated with higher grade of urothelial carcinoma [23]. Similar reported results by Hamdi, 2018 showed an evident association between high Bcl-2 expression and low grade carcinoma [24]. Additionally, another study done by Mohamed, 2016 demonstrated high association between Bcl-2 immunoreactivity and low-grade tumor and early tumor stages [25]. However, a study done by Fraile et al. 2003 showed different results that considered loss of Bcl-2 was not correlated with grading, staging or recurrence of urothelial carcinoma [26]. In the present study there were no significant association between Bcl-2 expression and DFS as well as OS was detected. On contrast, a study done by Wolf et al. 2001 showed that Bcl-2 positivity was associated with decreased tumor-free survival [27]. Based on our results that are accordance with most of the previous mentioned studies, it is possible to suggest that the significance of loss of Bcl-2 expression could help in identifying patients with worse prognoses. However, since the relationship between Bcl-2 expression and the biological behavior of cancer as well as its prognosis appears to vary greatly between societies, the utility of Bcl-2 in predicting cancer behaviors should be based solely on the society. These discrepancies can be because of the patient's characteristics or the antibody's specifications. Furthermore, our evaluation was limited by the small and monocentric population we used. Additional research with larger sample sizes is recommended.

According to recent research, FOXP3 is expressed in a range of tumours and has variable functional roles, contrary to the initial belief that it is an expression molecule restricted to Tregs. Its tumoral expression was detected in epithelial cancers of the breast, prostate, and bladder has been identified [28].

To the best of our knowledge, there are only few reports regarding the expression of FOXP3 in tumor cells. In this study, tumoral FOXP3 was expressed in 66.6% of all studied cases. Our results demonstrated that positive tumoral FOXP3 expression was significantly associated with lymph node metastasis, LVI and higher grade. These findings align with a study done by Zuo et al. 2007 which demonstrated a good association of positive tumoral FOXP3 with lymphatic metastasis, advanced stage and high proliferative index (Ki-67≥14%) in cancer breast [29].

Regarding the prognostic role of FOXP3 in tumor cells, we reported a significant association between expression and poor OS, which is in agreement with other studies that reported expression was linked to a decreased survival time [16, 28, 30, 31]. These studies found that increased expression of FOXP3 was associated with unfavorable prognosis in pancreas, bladder and breast carcinoma respectively.

In this study, our findings are in accordance with the results of study done by Winerdal et al. 2011, this study was the first one that examined FOXP3 expression in tumoral cells of urothelial urinary bladder cancer (UBC), as well as to its expression in tumor-infiltrating lymphocytes (TILs). Their results showed that patients with FOXP3+ tumor cells had decreased long-term survival compared to those with FOXP3–tumors [32]. Furthermore, our results are in align with another study done by Zhang et al. 2018 who showed that positive Foxp3 (exon 3-deleted isoform FOXP3Δ3) expression in the bladder epithelial cells inversely correlated with survival following radical cystectomy and promoted resistance to chemotherapy [28]. Additionally, another study done by Thoma, 2016 reported positive FOXP3 expression of this isoform increased with tumor stage in patients with bladder cancer [33]. These results indicate that FOXP3 expression in tumor cells, could be an important prognostic factor in UBC.

In the light of previous findings, targeted treatment directed at various FOXP3 sources may prove beneficial. First, FOXP3 splice variants that are expressed in tumours cells may act as biomarkers to aid medical professionals in making an earlier cancer diagnosis. Antisense oligonucleotides targeted cancer therapy may benefit from the use of Antisense oligonucleotides (ASOs) that impede FOXP3 pre-mRNA splicing as a means of influencing Tregs or tumour cells [34]. Because FOXP3 alternative splicing controls FOXP3+ cells' ability to repress, proliferate, maintain their lineage, and develop.

In conclusion, Loss of Bcl-2 expression and positive tumoral FOXP3 expression may be useful markers for urothelial bladder cancer patients with poor prognoses, making them possible targets for cancer treatment.

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