Clinical Significance of *BRAF* and *ZEB2* Expressions in Healthy Adjacent Tissue of Bladder Cancer

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Abstract

Background: Numerous molecular changes are involved in the development and progression of bladder cancer. Regular follow-up of patients is crucial due to the high recurrence rate of bladder cancer. The aim of this study is to determine the role of B-Raf proto-oncogene, serine/threonine kinase and *ZEB2* expressions in onset and progression of bladder cancer. We have also investigated their relationships to pathological characteristics.

Methods: We conducted this case-control study on bladder cancer and its healthy adjacent tissue, and normal bladder tissue from patients with benign prostatic hyperplasia. After extraction of total RNA and cDNA synthesis, quantitative expression analysis was performed in duplicate using real-time PCR. Changes in the gene expression were calculated according to the $2^{(-\Delta\Delta Ct)}$ equation. The products were confirmed by 1% agarose gel electrophoresis and sequenced by Bioneer Company. Data was analyzed using the SPSS software (version 16).

Results: There was significantly greater B-Raf proto-oncogene, serine/threonine kinase expression in 82% of bladder tumor samples compared to the adjacent tissues. In 91.1% of tumor samples, the gene expression was also significantly higher than healthy bladder tissues from patients with benign prostatic hyperplasia. We observed overexpression of B-Raf proto-oncogene, serine/threonine kinase in 61.7% of the healthy margin tissue samples compared to healthy bladder tissues of patients with benign prostatic hyperplasia (*P*<0.001). Expression of *ZEB2* in 52.9% of the bladder tumor samples was significantly higher than healthy peripheral tissues. This increase was observed in 94.1% of tumor samples compared to healthy bladder tissues of patients with benign prostatic hyperplasia (*P*<0.001). Pearson correlation coefficient showed a positive relationship between B-Raf proto-oncogene, serine/threonine kinase and *ZEB2* in cancerous samples (r = 0.75) and healthy margin tissue samples (r = 0.49).

Conclusion: During the carcinogenesis process, molecular changes are seen in healthy margin tissue. These molecular changes may be the reason for the high recurrence rate of bladder cancer. B-Raf proto-oncogene, serine/threonine kinase can potentially be a target cancer therapy in antisense technologies.

Keywords: Biomarkers, *BRAF*, *ZEB2*, Urinary bladder neoplasm

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Introduction

The occurrence and progression of bladder cancer (BC) is the result of a complex process of cancer cell growth from normal epithelial cells caused by changes in oncogenes, tumor suppressor genes, and genes involved in cell cycle, apoptosis, and repair of damaged DNA.[1-4] Numerous molecules are involved in these changes and may be used as diagnostic molecular markers for tumor growth and development.[5] Early diagnosis and regular follow-up of BC is essential due to its high recurrence rate.[6] Therefore, finding markers that have high sensitivity and are non-invasive can improve a patient's quality of life.[7] The B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) gene is one of the genes involved in cancer pathogenesis, which activates the MAP kinase/ERKs pathway and affects the pathways of cell proliferation, differentiation, and migration. The mutation in this gene is associated a number of diseases, including various cancers.[8] The inaccurate activity of the *ZEB2* gene has a role in induction of cell proliferation, epithelial–mesenchymal transition (EMT), migration, and apoptosis.[9] Mutations are common in the *BRAF* gene and the expression of the mutated gene is common among various cancers, such as melanoma, papillary thyroid, colorectal, and ovarian cancers.[10] Previous studies have found that none of BC samples had mutations in the *BRAF* gene.[11] In addition, mucus membrane with apparently normal tumor margins may acquire the characteristics of malignant cells at the molecular level.[12] The aim of this study is to determine the expressions of *BRAF* and *ZEB2* genes in BC tissue to its healthy marginal tissue and healthy bladder tissue of patients with benign prostatic hyperplasia (BPH) to determine their role in the onset and progression of carcinogenicity. We also investigated their potential use as diagnostic and prognostic biomarkers, and their relationship with pathological tumor characteristics.

Materials and Methods

Sample size, inclusion and exclusion criteria

According to a study by Zaravinous et al.[13], we evaluated 34 cases for the current case-control study. The Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1394.432) approved the study protocol, and written informed consent was obtained from participants after they received explanations about the purpose of the study.

Inclusion criteria consisted of patients with epithelial BC from all age groups and patients with BPH, which were matched for age (± 4 years) with the case group. In this study, participants were excluded if they had a history of chemotherapy or radiotherapy, cancers from other organs of the body, were undergoing chemotherapy or radiotherapy, patients with BC who were under any type of treatment, and the presence of a urinary tract infection.
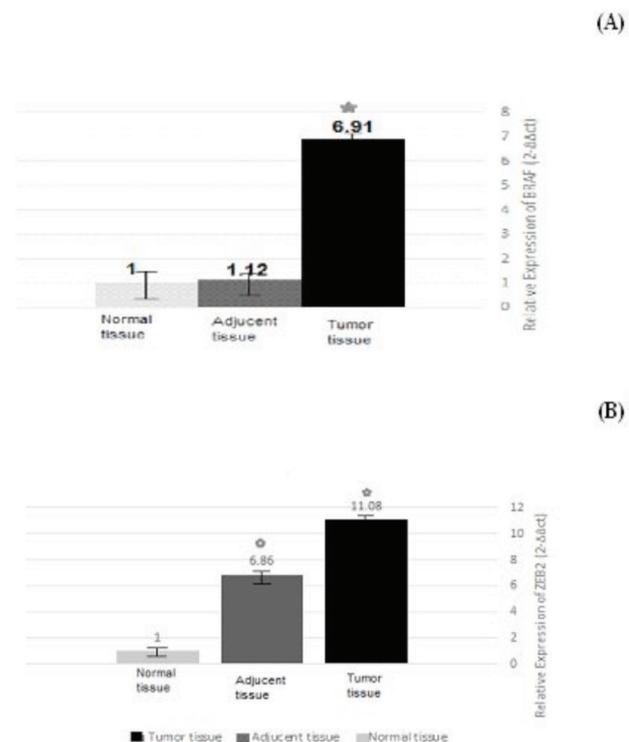

Figure 1. Comparison of the expression of the studied genes in tumor, adjacent tissues, and normal tissue.

Sample collection

We performed this study on BC specimens and its healthy marginal tissue from 34 patients, and healthy bladder tissue from 20 BPH patients who were diagnosed and treated at Shahid Beheshti and Bu-Ali Hospitals of Hamadan during 2013-2015. The method of preparation and storage of the specimens was previously described.[12]

The mean age of the studied population was 71 years. The highest age group was 60-80 years that comprised 79.4% of the total population. Two expert pathologists assessed all tissue samples. Patients were divided according to malignancy grade as follows: two cases had grade I [papillary urothelial neoplasm of low malignant potential (PUNLMB)] disease; 15 cases were grade II [low grade carcinoma (LG)]; and 17 cases had grade III [high grade carcinoma (HG)] disease.

All patients were monitored by urine cytology and ultrasonography every 3 months until the end of 2016. Bladder surgery was performed when there was an abnormal cytology or suspicious mass. Of the total 34 patients, 16 cases relapsed and four cases passed away.

RNA extraction

Total RNA was extracted by using the TRIzol reagent (Invitrogen, 15596-026). The concentration and purity of RNA was determined with nanodrop. The quality of extracted RNA was determined by electrophoresis on a 1% agarose gel.

Synthesis of cDNA and quantitative real-time PCR

We synthesized cDNA from RNA with a RevertAid First Strand cDNA Synthesis Kit (Fermentas, k.16222). After cDNA synthesis to reproduce the desired piece and quantitative evaluation of *ZEB2* and *BRAF* genes, we performed real-time PCR according to the SYBER Green method. Quantitative evaluation was performed in duplicate using primers designed by AlleleID 6 software (Table 1) and SYBR® Premix Ex TaqTM (Takara, RR820L). 18s rRNA was used as a reference gene, and we calculated the changes in gene expression according to the $2^{(-\Delta\Delta Ct)}$ equation.[14, 15] The products obtained from real-time PCR were transferred to a 1% agarose-gel and sequenced by Bioneer Company. The sequenced products were compared with the sequences in GenBank database.

Statistical analysis

The data were analyzed with SPSS software (version 16). In this study, a significance level of $P<0.05$ was used for all tests. We used the independent t-test to examine the relationship between the genes and parameters of age, sex, tumor stage, and size. The Pearson correlation coefficient was used to investigate the relationship between genes.

Results

Expressions of the B-Raf proto-oncogene, serine/threonine kinase (BRAF) and ZEB2 genes in the studied groups

There was significantly higher *BRAF* expression in 82% of BC samples compared to healthy marginal tissues. In 91.1% of tumor samples, we observed significantly higher gene expression than healthy bladder tissue from BPH patients. In 61.7% of the samples, there was increased expression of the *BRAF* gene in healthy marginal tissue compared to healthy bladder tissue from BPH patients ($P<0.001$). There was significantly higher *ZEB2* expression in 52.9% of the BC samples compared to healthy peripheral tissues. This increase was observed in 94.1% of tumor samples compared to healthy bladder tissue from BPH patients ($P<0.001$; Figure 1).

As shown in Figure 1A, the *BRAF* gene expression increased in tumor tissue (6.91-fold) and healthy marginal tissues (1.12-fold) compared to normal tissue. Figure 1B shows an increase in *ZEB2* expression in cancerous (11.08-fold) and marginal tissue (6.86-fold) compared to normal tissue.

Table 1. Primer sequences and NCBI accession number of studied genes.

NCBI accession	number	Forward primer	Reverse primer
18s rRNA	X03205.1	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
ZEB2	NM_014795.3 NM_001171653.1	GGAACCAGAATGGGAGAAACG	CAAGGAGCAGGTAATCGCAAGT
BRAF	NM_004333.4	GGTGATGTGGCAGTGAAAATG	CAGTTGTGGCTTTGTGGAATAG

Relationship between clinical and pathologic characteristics with expressions of ZEB2 and B-Raf proto-oncogene, serine/threonine kinase (BRAF)

Table 2 shows the relationship between clinical and pathological characteristics. According to the results, there were significant differences between *BRAF* expression in individuals who were exposed to carcinogens and those without exposure to carcinogens (*P*=0.03).

Correlation between expressions of the studied genes

Pearson correlation coefficient results showed a positive correlation between the *BRAF* (0.75) and *ZEB2* (0.49) genes in the cancerous samples and marginal tissues. There was no correlation between *ZEB2* and *BRAF* in normal tissue (r=0.122).

Discussion

The exact mechanism of BC and its recurrence has not been clearly identified until now. Therefore, it is a necessary requirement to understand the process of molecular changes for finding biological markers and therapeutic goals.[16] In this study, we have evaluated the level of mRNA for the *ZEB2* and *BRAF* genes in BC samples, adjacent tissue, and healthy bladder tissue in patients with BPH. The results of this study indicated that the expression of *BRAF* in tumor samples was higher than that of healthy margins and healthy bladder tissue in patients with BPH. *BRAF* had higher expression in healthy marginal samples than healthy bladder tissue of patients with BPH. Unlike our findings, Zaravinos et al. reported that *BRAF* expression in tumor tissues did not significantly differ from healthy marginal tissue. They observed that 63.3% of the samples had the same expression.[13] In other studies on papillary thyroid cancer, the increase in *BRAF* gene expression was introduced as a predictor of metastasis in cancer. Its expression in malignant nodules was higher than benign nodules.[17, 18] The results of this study indicated an increase in *ZEB2* expression in tumor samples compared to marginal tissue and normal tissue, which supported the findings of Liu et al.[19] The amount of the *ZEB2* protein is a key factor in regulating cancer cell invasion, which significantly increases in BC compared to the adjacent tissue.[20] There is increased expression of the *ZEB2* gene in stomach, ovarian, and pancreatic cancers.[21] However, normal bladder tissue has not been introduced as a control group in the mentioned studies. Therefore, the results do not reflect molecular changes in tumor and adjacent tissue rather than normal tissue. According to the design of the current study, we have observed increased expressions of *ZEB2* (11.08-fold) and *BRAF* (6.91-fold) in tumor samples compared to normal bladder tissue from patients with BPH. There was a 6.86-fold increase in *ZEB2* expression and 1.12-fold increase in *BRAF* expression in adjacent samples compared to healthy bladder tissue from BPH patients. This finding indicated that the molecular changes in the cancer process affected the healthy marginal tissue, and these molecular changes might be the reason for the high recurrence rate of BC. Therefore, surgical removal of the tumor along with its marginal tissue could not be considered a complete treatment method. Chemotherapy or immunotherapy might be recommended in addition to surgery. In this study, there was no significant relationship between the expressions of *ZEB2* and *BRAF* with clinical and pathological characteristics of tumor size, tumor grade, muscle invasion, mortality, and smoking. In a study conducted by Lee et al., there was no

significant difference in *ZEB2* expression in BC tissue between PUNLMP grade and LG. In addition, they observed no significant difference between LG and HG,[22] which was consistent with the findings of this study. In contrast, Xue et al. reported that *ZEB2* has a role in muscle invasion.[23] In a Cai et al. study, there was no significant correlation between *ZEB2* expression, tumor size, and tumor grade in liver cancer, which was consistent with our findings.[24] In patients with PTC, it was found that recurrence and metastasis to other sites were caused by increased expression of *BRAF*.[25] Bhandaru et al., in their study on melanoma skin cancer, reported that increased tumor size and progression in tumor TNM staging were associated with increased *BRAF* expression.[26] Fang et al. reported that there was no relationship between *ZEB2* gene expression and characteristics such as gender, age, tumor size, tumor grade, and tumor stage in kidney cell carcinoma.[27] The difference in our findings with other studies might be attributed to the difference in this disease among various populations. In addition, BC is a multi-factorial and epigenetic disease. Ultimately, the differences in sample size and trend process of carcinogenesis in various organs might result in differences. The advantage of our study compared with previous studies was the presence of two types of control groups and investigation of the gene expression in each individual sample. Due to the variations in tumor cell characteristics at the molecular level, we used two types of adjacent tissue and healthy normal tissue as control groups. In addition, the two control groups were used to eliminate the confounding factors, which could be effectively used to reject or confirm the results.

In the study of the relationship between gene expression and exposure to carcinogenic substances, it was found that the *BRAF* gene had a significant increase in 100% of people with exposure to carcinogenic substances. In 30% of subjects, there was an increase in *ZEB2* gene expression, which was not statically significant. In a study by Wang et al. on a lung cancer cell line exposed to arsenic, the group with reduced p53 showed increased *ZEB2* expression, while in the group without change in p53 and exposure to carcinogenic substances did not affect the induction of *ZEB2* gene expression.[28]

Carcinogens may cause changes in various molecules in different signaling pathways such as the cell cycle, proliferation, apoptosis, and angiogenesis. Although there are no findings in this area of BC, cigarette smoking can cause a change in the expression of some pathways such as the WNT/B catenin pathway in chronic pulmonary obstruction.[29]

The results obtained from the Pearson correlation coefficient showed a positive, direct effect of *BRAF* expression on the *ZEB2* gene in cancer specimens and adjacent tissues. This relationship was not observed in normal tissue. The *BRAF* gene is located on chromosome 7 (7q34) and it is a protein that encodes the raf/mil family of serine/threonine kinase proteins. This protein plays a role in the signal pathway of MAP kinase/ERKs, which affect cell division and differentiation.[8] The interaction of RAS-GTP in the membrane stimulates the activity of RAF kinase, which results in phosphorylation of MEK1 and MEK2. Then, the activated MEK1 and MEK2 lead to activation of ERK1 and ERK2. In this regard, this phenomenon transmits the signal into the nucleus and regulates cell proliferation, differentiation, and migration.[30] Among the RAFs, *BRAF* is the strongest activator of the MAP/ERK/MEK signaling pathway.[17] Increased *BRAF* expression increases the phosphorylation of ERK1/2 as a result of the activation of the MAPK signal pathway.[31] *ZEB2* is located on chromosome 2q22.3, which is an encoding protein in the nucleus and is known as a DNA-attached transcriptional inhibitor that is activated with Smad.[32] The reduction of *ZEB2* expression is associated with a decrease in the expression of CDK4/6, cyclin D1, cyclin E, E2F1, and c-Myc along with an increase in the expressions of p15 and p21. It also increases E-cadherin and inhibits the expressions of β-catenin, vimentin, N-cadherin, and snail. Disabling and deleting *ZEB2* expression can inhibit proliferation, migration,

Table 2. Relationship between exposure to environmental carcinogens, muscle invasion, tumor grade, tumor size, recurrence, and expression levels of *ZEB2* and B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) genes.

Variable		Number	BRAF		ZEB2	
			Mean ± SD¶	*P*-value	Mean ± SD	*P*- value
Muscle invasion	MIBC¶¶	13	11.6 ± 3.03	0.9	13.03 ± 3.05	0.8
	NMIBC¶¶¶	21	11.6 ± 2.4		13.29 ± 3.2	
Grade	PUNLMP●	2	13.2 ± 0.1	0.1	15.6 ± 0.07	0.3
	LG●●	15	11.03 ± 2.6		12.2 ± 3.3	
	HG●●●	17	12.2 ± 2.7		13.8 ± 2.8	
Smoking	Yes	22	11.7 ± 2.4	0.9	13.5 ± 3.05	0.4
	No	12	11.8 ± 3.1		12.6 ± 3.2	
Carcinogen Exposure	Yes	10	12.2 ± 1.5	0.03	14.1±2.1	0.056
	No	24	11.6 ± 3.02		12.8 ± 3.4	
Size	<10 cm2	10	11.3 ± 3.07	0.5	13.2 ± 3.5	0.9
	>10 cm2	24	11.9 ± 2.5		13.3 ± 2.9	
Recurrence	Yes	16	12.11 ± 2.09	0.14	13.9 ± 2.4	0.08
	No	18	11.47 ± 3.11		12.66 ± 3.6	

●PUNLMP: Papillary urothelial neoplasm of low malignant potential; ●●LG: Low grade; ●●●HG: High grade; ¶SD: Standard deviation; ¶¶MIBC: Muscle invasive bladder cancer; ¶¶¶ NMIBC: Non-muscle invasive bladder cancer

and invasion, and induce cell apoptosis.[9] *ZEB1* and *ZEB2* via the TGFβ, NFKB, and NOTCH signaling pathways play an important role in tumor progression through the EMT process.[33] Therefore, *BRAF* can be a potential target therapy for antisense technologies, which is also more effective by directly targeting *ZEB2*. The purpose of such studies is to locate molecular targets for the treatment of cancers.[8] BC is cumulatively being seen as a disease that cannot be managed solely on the basis of pathologic staging; the synergy among various factors that target different pathways to achieve optimal response and the use of therapeutic regimens that targets various molecular pathways may be a key to successful treatment.[34] Increased expressions of these genes in tumor tissue and healthy marginal tissue indicated their oncogenic role in BC. Based on this study, these genes were presumed to be suitable biomarkers for the diagnosis or probable prediction of recurrence, but not useful for evaluation of the pathological characteristics of BC. Due to the limitations in the present study, only small populations were evaluated. Further studies in these areas would be necessary to increase the accuracy of the results and understand the relationship between expression of these genes and clinicopathologic characteristics such as invasiveness and tumor degree.

Conclusion

During the carcinogenesis process, molecular changes are seen in healthy marginal tissue. These molecular changes may be the reason for the high recurrence rate of BC. With regards to the positive and direct effects of *BRAF* expression on the *ZEB2* gene in cancerous and adjacent tissue specimens, and the lack of relationship in healthy tissue, we have proposed that *BRAF* could be a targeted cancer therapy in antisense technologies.

Acknowledgement

Conflict of Interest

None declared.

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