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COMPUTATIONAL IDENTIFICATION OF UPREGULATED GENES IN BREAST, PROSTATE AND COLORECTAL CANCER AND THE CELLULAR SIGNALING PROCESS

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ABSTRACT: Cancer is considered as the deadliest disease all around the globe as it imparts a massive death toll among the public of all races, sexes and age groups. According to SEER and IARC, 14.2 million new cases of cancer are diagnosed annually. The annual rate in developed countries is 6.2 million (45% of the total cases) along with mortality rate of 3.0 million (36% of the total cases). In Developed countries, the rate of incidence is 9.0 million (60% of the total) and the mortality rate is 5.5 million (67% of the total). The prime focus of the study was to utilize the computational approaches for the identification of the genes those are upregulated in breast, prostate and colorectal cancers. Furthermore, it was also aimed to study the cellular signaling process involved in gene upregulation. This study was conducted in National Hospital Hong Kong. Total of 130 respondents were considered and their samples were collected. These samples were then subjected to PCR and the results revealed that there were certain genes those were upregulated in all three types of cancers. OX genes were significantly targeted as its upregulation strongly affects the normal cellular functions. This study not only helped in identification of upregulated genes but also led us to invent new control measures against cancer.

Keywords: Cancer, Computational, breast, colon, colorectal, prostate, OX genes.

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1.INTRODUCTION

Major proportion of the world population is on the verge of several deadliest diseases including Hepatitis, Diabetes and several other contagious diseases. Of all these deadliest illnesses, Cancer is the most prominent and serious disease which impose serious threats to human lives. It is until now, considered as incurable disease as there is no such remedy has been discovered all around the world [1-6]. Cancer itself is not the name of a single disease but is a complete set of diseases in which the cell cycle is totally disrupted, and cells continue to proliferate or invade all other parts of the body. Categorization of cancer is mainly based on the type of the cell and origin of the tumor. Classification of cancer depends primarily on the cell type and origin of the tumor; cancer can be classified as carcinoma, sarcoma, or lymphoma based on whether the tumor is derived from epithelial cells, connective tissue, or lymph nodes, respectively. Cancers with the highest incidence are lung, breast, stomach, prostate, colorectal, and uterine cancer. Of the total identified cancers all around the world, only breast cancer accounts for 14% in females which makes it world's 2nd deadliest disease. It is a genetic disease and is resulted by mutations in different genes that are responsible for several metabolic pathways involved in the cell cycle. The disease symptoms include distorted breast shape, fluid released from the nipples, skin color is diminished on the nipples and scaly, reddish skin on the nipple. In most cases approximately 44% cases are regarded as asymptomatic in which no symptoms of the disease occur. The UK-based screening method, according to the evidence provided by Threlfall et al. reduces BC mortality [7-12]. Accumulated research bodies have revealed the projected rate of cancer that might increase up to 19.5 million in 2025 [13-18]. About 1.8 million new cases of breast cancer are reported annually. According to the American Cancer Society (ACS), about 2-3% deaths arise from breast cancer each year [19-26]. It is the most frequently diagnosed cancer in the world i.e., 25% cases in the USA. In Asian countries, especially in Pakistan more than 45,000 Pakistani females are starved to death due to breast cancer and rate of incidence approaches to every single female out of 9, which is alarming for less developed countries like Pakistan [27-33]. The present form of therapeutic options for cancer has improved patient's life expectancy. But still there is need to devise the new targets and their mechanistic base of action. In the past, the coding region of the human genome and its role in tumor biology has been focused. RNA acts as an ambassador between gene and final product hence coding regions are considered to play an important part in gene expression and gene regulation [34-42]. However, with the advancement of technology, science has helped in the discovering the transcriptional units called ncRNAs that include IncRNAs and small noncoding RNAs, short interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), (Piwi-interacting RNAs (piRNAs), and microRNAs (miRNAs). These RNAs are present in intra /inter-genic regions of the genome and hold organizational, efficient, catalytic and gene regulatory role. miRNA is one of such ncRNAs is not only involved in the development of cell and its differentiation processes, but also plays a

decisive role in carcinogenesis [43-48]. With an estimate of 165000 novel cases and 30,000 deaths in 2018, Prostate Cancer (PCa) is the most prevalent cancer in men. Furthermore, it is the second major leading cause of cancer death in males in the United States [49-56]. As the Androgen Deprivation therapy is regarded a standard treatment of prostate cancer, yet PCa has developed resistance to the previously described intervention. The developed tumor become uncontrolled and develops into a more aggressive form which is called as Castration- Resistant Prostate Cancer (CRPC) [57-62]. Recent studies suggest that the upregulation of MAPK/ERK torrent is responsible for the transition of androgen-independent condition to the prostate cancer. However, oncogenic mutations in receptor tyrosine kinases (RTKs) are not so common and the molecular mechanism that leads to aberrant MAPK activity in male prostate cancer is still unidentified [63-66]. These explanations propose the loss of physiology of key negative regulators of the pathway including DAB2IP and SPRY2 genes, which encode intracellular antagonists of RTKs signaling [67-71]. Another type of Cancer, colorectal cancer (CRC), is a disease manifested by the abnormal behavior of cells that proliferate and destroy pre-existing tissues, both locally, at the site of origin and other body parts, i.e., metastatic regions. This self-centered cell growth, and varied metabolic activities allied with it, can be drastic, despite of medical treatments. The anomalous behavior of tumor cells is obsessed by variations in cell biology and tend to alter critical phenomenon such as proliferation, invasion, evasion to cellular death, and escape to immune surveillance, i.e., entitled hallmarks of cancer [71]. Such alterations in cell biology, are regarded as the outcomes of an evolutionary process through which gene mutations and somatically obtained copy number alterations (CNAs) accumulate and result in the selective advantage of cells that carry such alterations. The aggregation of genetic destruction results in the expansion of initially benign (i.e., non-invasive) tumors, which, when untreated, form invasive subclones and lead to cancer. DNA alterations come in many flavors, namely, small nucleotide variants (SNVs), small insertions or deletions (Indels), structural variants (SVs) or epigenetic alterations, mostly promoter hypermethylation, and in chromosomal copy number alterations, i.e., aneuploidy. The role of chromosomal aneuploidy in tumorigenesis was for a long time underestimated despite observations by Hansemann in the 19th century [72], and work by Theodor Boveri in the 1920s . In fact, in several hematological neoplasia and soft tissue tumors, specific chromosomal rearrangements had been found to be pathogenic. The process of Karyotyping of metaphases from tumor cells has been a key for such observations, and this strategy proved well in leukemias and soft tissue tumors, describing single or few chromosome rearrangements resulting in fusion genes . Though, the role of definite chromosomal aberrations in the origin of solid tumors like CRC was tough to develop, mainly characteristic to very complicated and highly organized karyotypes. Although, using such techniques like DNA flow cytometry and DNA image cytometry, it was clearly determine that most CRCs showed abnormal nuclear DNA content, because of such chromosomal copy number alterations can be the only explorations [73] Despite of significant

progress in the field of surgery, the overall endurance ratio of patients suffering from cancer is still inacceptable, which need novel strategies in cancer therapy. New recommendations for cancer therapy comprise of Radiotherapy and Chemotherapy to effectively kill cancer cells and also, alleviate the reappearance of cancer, a spectacle which is common after surgery. Luckily, a variety of chemotherapeutic agents have been devised for cancer remedy and they have explicated a great anti-tumor potential . Cisplatin, paclitaxel, docetaxel, doxorubicin, gemcitabine and oxaliplatin (OX) are among the most common drugs applied in chemotherapy. OX is a diamino cyclohexane-containing third-generation platinum compound that was first identified in 1976, but its application in cancer therapy was approved 20 years later in 1996 [49-57]. OX can be applied in cancer therapy as mono-substrate, but in order to promote its antitumor activity, it is extensively used with other anti-tumor agents[74]

2. MATERIALS AND METHODS

2.1. Sample collection

From February 2020 to February 2021, prostate tissue samples were gained from patients who were administered to National hospital in Hongkong. All the patients were newly reported cases with no previous medical history of surgery or chemotherapy. A written consent was signed by every single patient by the Ethical Committee of the National University of Medical Science (Hong Kong). Cases comprised of 130 tissue samples, including 45 prostate cancer/ matched normal tissue and 35 benign prostate hyperplasia (BPH) samples, used as non-neoplastic controls. Prostate glands obtained by radical prostatectomy and BPH tissues obtained by transurethral resection were quickly transmitted to the pathology lab of National Hospital. Highly trained and expert genitourinary pathologist parted the cancerous and adjacent tissues that did not contain cancer and hyperplastic tissues in BPH cases in two replicates, one of them was assigned for the evaluation of histopathological features of the tissue and the other was swiftly engrossed in RNA later solution (QIAGEN, Germany). They were stored at standard room temperature for a period of 24 h, and finally shifted into a freezer at -80 temperature for storage. tissues were defined as cancerous, normal or BPH after careful examination and confirmation of by the pathologist.

2.2. RNA extraction and cDNA synthesis

The total amount of RNA was extracted from a 100 mg sample of prostate cancerous tissues by using Tri Pure Isolation Reagent (Roche, Switzerland). The extracted RNA yielded (on average 500-550 ng of a total volume of 35 ml) and limpidness (average A260/A280 ratio 1.7) was enumerated with the help of Nano Drop 2000-C (Thermo Scientific, USA). 1100 nano grams of total extracted RNA from each collected sample was exposed to complementary DNA synthesis along with randomly arranged hexamers and Oligo (dT) primer. Finally, with the help of Prime-Script RT reagent kit (Takara, Japan) according to manufacturer’s recommendations, cDNA product was adopted as the template for RT-PCR.

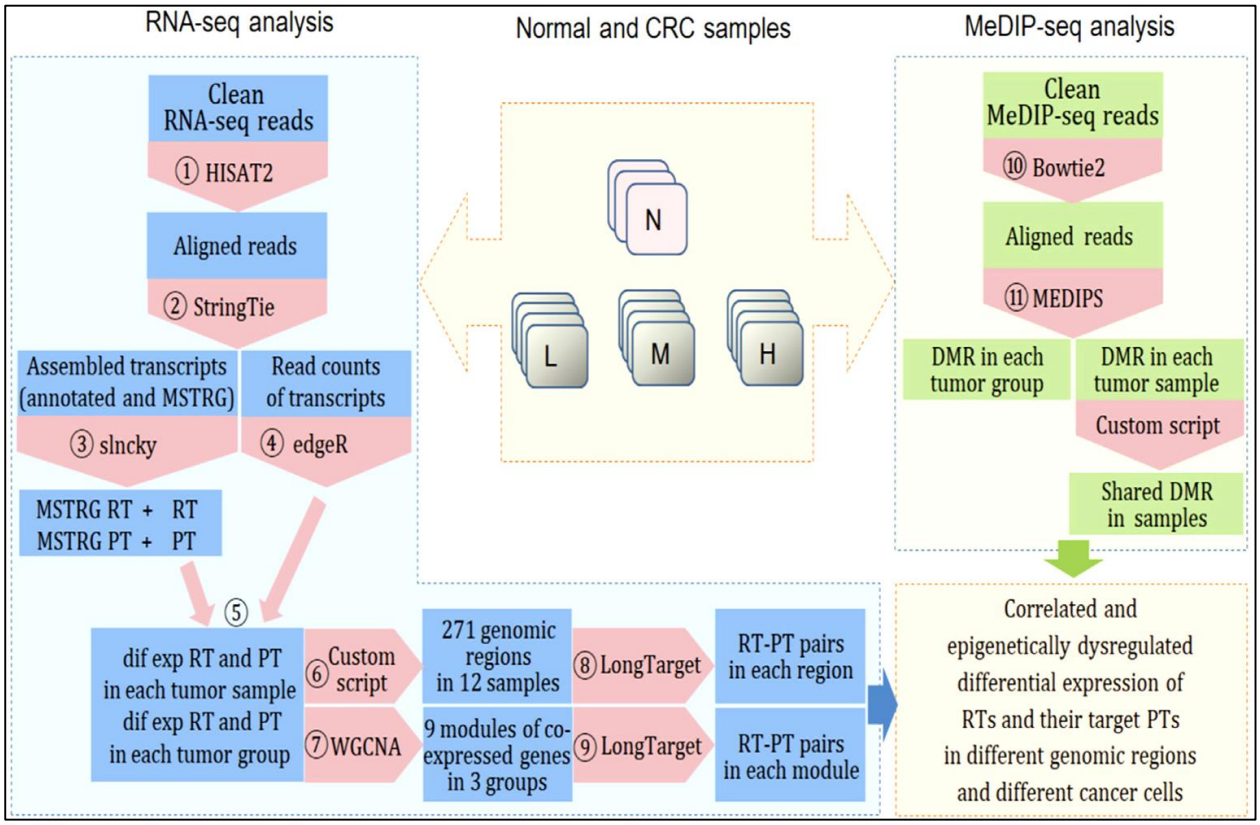

Figure 2.1. RNA extraction and cDNA synthesis from samples

2.3. Identification of DAB2IP and SPRY2 transcript isoforms

Exon-exon spanning primers were considered specially to amplify DAB2IP transcript 1 and 2, SPRY2 transcript 1, 2, 3 and 4. PGM1, β-actin and GAPDH were previously reported to be normally expressed in various human tissues, were tested and GAPDH was determined to act as the internal reference gene. Amplification was carried out in volumes of 21 µl, containing 1 µl of cDNA, 0.6 µl of primers (with a concentration of 10-11 pmol) and 15 µl of master mix. After primary denaturation of primers at 94 °C for 4-5 min, the thermal cycling was outperformed as the following for 35 cycles; 95 °C for 35 s, 62 °C for 32 s, 70 °C for 28 s and a final extension of 5 min at 75 °C. PCR amplified fragments were separated on a 1.5% agarose gel, containing Green Viewer.

2.4. Quantitative real-time RT-PCR

To quantify the expression of DAB2IP.1 and SPRY2.2, Real-Time PCR was performed in duplicate, using SYBR Premix Ex Taq II. The PCR amplification guidelines were strictly followed and the profile follows the sequence: denaturation for 30 s at 96°C; 34 cycles: 6 s at 94 °C, 32 s at 65°C, 35 s at 75°C. A melting curve was developed after each run which verified the purity of the primers. Messenger RNA (mRNA) expression in each tissue sample was standardized to the Ct values determined for GAPDH gene. Real-Time PCR assay was adjusted and carried out by a Rotor-Gene Q (QIAGEN GmbH) instrument. To assess the gene expression aberrations, the average Ct value of repeated samples was reflected.

2.5. Predicting the promoter region

To identify the promoter regions of the expressed transcript alternates, the sequence of 1 kb upregulated transcript start sites (TSSs) were analyzed. Data from the Encyclopedia of DNA Elements at UCSC genome browser was used to find relaxed chromatin structure near the promoters by analyzing the potential hypersensitivity to DNaseI, Tri-methylation of lysine 4 on histone H3 (H3K4me3) and acetylation of lysine 27 on histone H3 (H3K27ac).

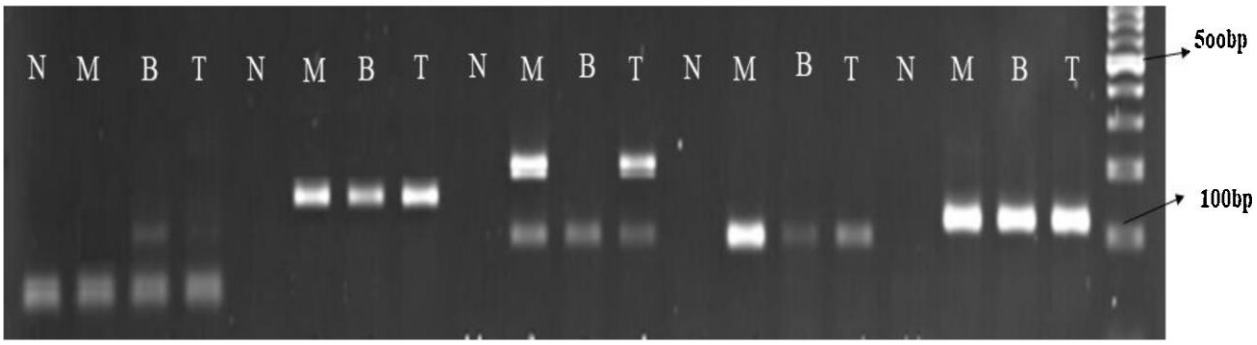

Figure 2.2. Identification of Promoter region reveals the genome prediction

The GP miner and Eukaryotic Core Promoter Predictor programs were utilized to discover the core promoter motifs like TATA box, GC box, initiator element (INR) and downstream promoter element (DPE). Additionally, for the identification of regulatory genes that remain conserved across all species, DNA sequences of different species were utilized from NCBI and aligned with the MAFFT multiple sequence alignment program.

Table 1. Demographic and pathological characteristics of the study participants

Parameter (%)	**PCa (%)**	**Matched Normal Tissue (%)**	**BPH (%)**	**Total (%)**
Patient Number	50 (38,4)	50 (38,4)	30 (23)	130
Age (Years) <60 >60	8 (16) 42 (84)	8 (16) 42 (84)	5 (16.6) 25 (50)	21 (16.1) 109 (83.6)
PSA (ng/ml) >4 4-10 <10	3 (6) 37 (74) - -	- - - -	28 2 - -	- - - -
Perineurial Invasion Negative Positive	41 (82) 9 (18)	- - -	- - -	- - -
Capsule	41 (82)	-	-	-

Invasion Negative Positive	9 (18)	- -	- -	- -
Lymph Node Metastasis Negative Positive	45 (90) 5 (10)	- - -	- - -	- - -
Gleason Score 6-7 8 9-10	37 (74) 5 (10) 8 (16) -	- - -	- - -	- - -

3. RESULTS AND DISCUSSION

3.1. Clinical and pathological characteristics

Clinical and pathological features of the study participants are shown in Table1. The mean age of PCa group and Standard Deviation (SD) were 65.67 ± 6.66 years with the Average of PSA 10.85 ± 8.85 ng/ml. The mean age of BPH group was 66.66 ± 11.99 years with the mean PSA of 4.03 ± 1.88 ng/ml.

3.2. Expression of DAB2IP and SPRY2 transcript variants in three groups

To investigate DAB2IP and SPRY2 transcript variants in human prostate cancer, RT-PCR analysis was carried out by utilizing transcript-specified primers. As depicted in (Fig. 1) DAB2IP.1 and SPRY2.2 shows different expression profiles among three groups, while other splice variants showed almost ubiquitous expression pattern in all samples. To quantify expression levels of DAB2IP.1 and SPRY2.2, Real-Time PCR was carried out and the melting curve was drawn. The amplification of the desired targets was confirmed by the presence of a single specific peak for each gene except for one PCa sample, which was an outlier and excluded from the study. Rest analysis showed that, the expression level of DAB2IP.1 in PCa was significantly lower compared to paired normal tissue (P=0.001) (Fig. 3.3).

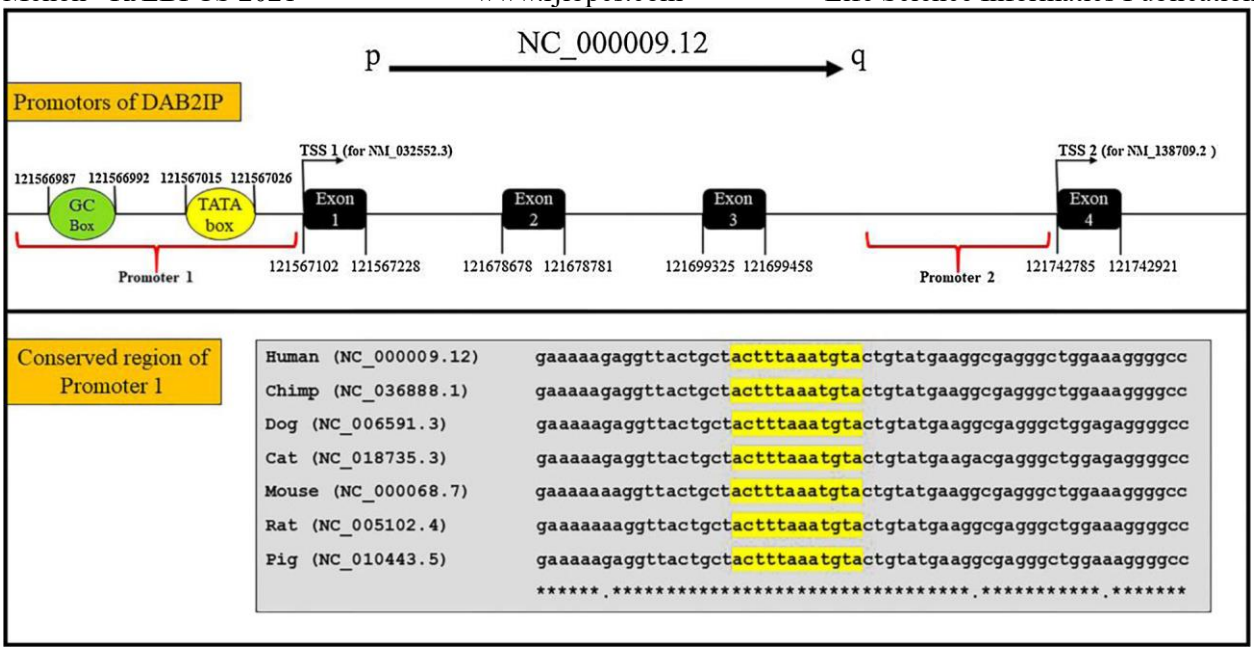

Figure 3.3. The schematic of the genomic organization of the human DAB2IP gene.

3.3. Association of qualitative demographic/pathological characteristics with DAB2IP.1 and SPRY2.2 levels

By following the principles as described by Mann-Whitney test, the association of qualitative demographic/pathological features with mRNA levels of DAB2IP.1 and SPRY2. quantified. The qualitative demographic/ pathological characteristics including gender, perineurial invasion, capsule invasion and lymph node metastasis did not correlate with the levels of DAB2IP.1 and SPRY2.2 expression.

3.4. Correlation of quantitative demographic/pathological characteristics

To assess the quantitative clinicopathological characteristics, association with DAB2IP.1 and SPRY2.2 expression level, Spearman's Rho test was conducted between two varied conditions of each characteristic. After excluding three patients sample whose PSA level was outlier, results showed that there was a significant negative correlation between DAB2IP.1 and pre-operative serum levels of PSA (P=0.039 ρ =−0.24), SPRY2.2 and pre-operative serum levels of PSA (P=0.045 ρ =−0.3). As well, there was a significant positive correlation between DAB2IP.1 and SPRY2.2 mRNA down-regulation in tumor samples (P=0.001 ρ=0.4323).

3.5. Functional promoter regions

The upstream region of the TSS in DAB2IP and SPRY2 was computationally analyzed to investigate the observed expression patterns of different transcript variants of DAB2IP and SPRY2. There were two potent promoter regions identified in DAB2IP gene and three in SPRY2 gene. Subsequently, the sequences related to these promoter regions were screened to determine the core promoter elements. The analysis of DAB2IP promoter regions revealed that the first promoter region was conserved across species and contained regulatory core promoter elements in contrast to the second promoter region. The investigation of SPRY2 promoter regions revealed that none of these regions

comprise the TATA box. However, both the first and the second promoter contain a GC box. Moreover, no regulatory element was ascertained in the third promoter region. In spite of this, the region of interest was situated in the first promoter region being conserved in all vertebrates. As this region holds a conserved CAAT box, the stable level of expression related to the first transcript variant of SPRY2 in all samples could be attributed to this element.

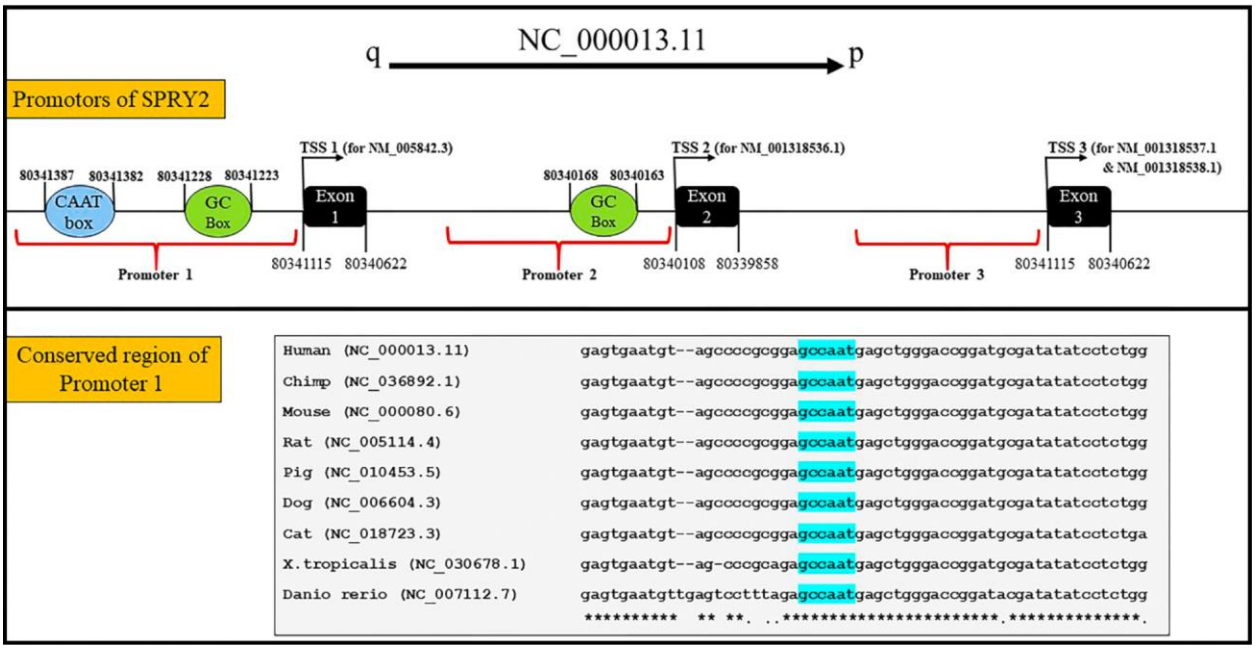

Figure 3.4. The schematic of the genomic organization of the human SPRY2 gene. SPRY2 transcript variants are generated by transcription start sites (TSS) from exons 1, 2 and 3.

4. CONCLUSION

The analysis of DAB2IP promoter regions revealed that, unlike the second promoter region, the first promoter region contained regulatory core promoter elements. Cross-species alignment of Human, Chimpanzee, Dog, Cat, Mouse, Rat, and Pig revealed that TATA and GC box elements are highly conserved across species. Black boxes indicate exons and for simplicity, the size of the exons is depicted in the same way. The schematic of the genomic organization of the human SPRY2 gene. SPRY2 transcript variants are generated by transcription start sites (TSS) from exons 1, 2 and 3.

Since the expression of DAB2IP.1 and SPRY2.2 did not show any differences in patients with perineural/capsule invasion, lymph node metastasis and Gleason score, and since DAB2IP.1 level was lowered in tumor than paired non-cancerous tissue and SPRY2.2 is reduced in tumor than matched normal tissue and BPH, it is inferred that DAB2IP.1 down-regulation may act as an early event in tumorigenesis and SPRY2.2 down-regulation may have a role in transition from benign to malignant condition, but not in tumor progression. Such patterns of correlation could indicate existence of tissue or condition-specific mechanism for regulation of expression of these genes which should be elaborated in future studies. In general, data obtained from the current study have confirmed the earlier reports on DAB2IP and SPRY2 up regulation in diverse human cancers.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The author confirms that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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