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Computational analysis of AP-2γ role in bladder cancer

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ABSTRACT

AP-2γ protein is a member of AP-2 transcription factors family, which participate in early developmental processes. Its oncogenic function has been confirmed in few cancers (e.g. breast, skin or ovarian) whereas the role in bladder cancer (BLCA) is not well understood. Nowadays, disruptions in pathways such as FGFR3/RAS or TP53/RB1 are the best known, yet new genes that would allow a better understanding of this heterogeneous tumor are being researched. The current literature indicates the promoting effect of AP-2γ encoding gene (*TFAP2C*) on distant recurrence or tumor growth. Similar relationship, according to which increase in *TFAP2C* expression significantly worsen prognostic endpoints, was observed by performing GEPIA on bladder cancer cohort. Using UALCAN platform, we noticed *TFAP2C* expression increase in bladder cancer compared to normal tissue. To assess the role of this gene in BLCA, GSEA analysis was performed regarding the AP-2γ targets on *TFAP2C* "high" and "low" groups. Beside interesting patterns related to groups distinction by genes whose expression profile is specific to BLCA, we found other curious results which we implemented for DAVID bioinformatic resources. Using functional annotation, we described selected gene sets having highest statistical significance. For *TFAP2C* high group we observed descriptors of many signaling pathways (e.g. PI3K-AKT, MAPK, ERBB) but also processes e.g. loss of adhesion, regulation of apoptosis, proliferation or cell cycle. For *TFAP2C* low group we matched the processes guided by miRNA pathways, verifying the literature data of AP-2γ interaction with RNA molecules. Within such, descriptions of processes revealed implication in cell differentiation, positive cell-to-cell communication or transcriptional misregulation in cancer. Conclusively, our analyzes outline the distinct consequences of various *TFAP2C* levels, simultaneously indicating the need for deeper reflection on the subject of *TFAP2C* participation in the BLCA.

INTRODUCTION

BLADDER CANCER

EPIDEMIOLOGY

Bladder Carcinoma (BLCA) is the tumor whose incidence has been decreasing over recent years. It is more common in men and is concurrently the fourth cancer in the frequency of occurrence among them (Cassell, 2019). However, cancer-related deaths are stable in men, whereas slightly reduced over the last years in women. According to the American Cancer Society, estimates for the year 2020 concerns about 81,400 new cases of cancer within which 17,980 as fatal (American Cancer Society, 2020). Regarding the newest Polish statistics, in 2017 the incidence of new bladder cancer cases exceeded 5,770 among men (Wojciechowska, 2017).

CLASSIFICATION

Using information from the https://www.cancer.gov website, the bladder cancer can be divided into transitional and squamous cell carcinomas, or adenocarcinoma. These three types are established depending on which cells lining the bladder are transfor-ming into cancerous. Apart from the classical histological division, along with the development of techniques in a branch of biochemistry, it is also possible to distinguish molecular types. University of North Carolina (UNC) demarcates luminal or basal bladder cancer. In contrast, three groups (basal, luminal and TP53-like) are dis-tinguished by MD Anderson Cancer Center (MDA) and five groups by The Cancer Genome Atlas (TCGA) consortium (Inamura, 2018). The last two are in agreement since the main three subtypes defined by TCGA correspond to the one summarized by MDA, concisely characterized in the following subsections. Finally, the latest type of distinguishment appears to be the Lund classification system (Aine, 2015) which considers e.g. different transcription factors, indicating their importance in such heterogeneous disease. Since the prediction of targeted treatment response is determined by molecular subtypes (Aine, 2015), it is reasonable to collate the current state of knowledge about the molecular background along with a more thorough consideration of the aspect of transcription factors for a broader view of alterations at the cellular level.

Basal bladder cancer

The first main molecular subtype of urothelial carcinoma is characterized by a resemblance to cancer stem cells (CSCs) and possessing expression of biomarkers typical for epithelial-to-mesenchymal transition (EMT). Human xenografts metastasize more widely and take advantage of the EMT-dependent mechanism more profusely compared to luminal type (McConkey, 2016a). Expression signature in this tumor consists of Annexin-1, cluster of differentiation 49 (*CD49*), epidermal growth factor receptor (*EGFR*) and Cyclin B1 proteins upregulation (Dadhania, 2016). Nevertheless, better sensitivity to neoadjuvant chemotherapy (NAC), in contrast to luminal type, was confirmed in the literature (McConkey, 2016b).

Luminal bladder cancer

In this type of tumor, there is a similarity to normal urothelium (both intermediate and superficial layers) (Dadhania, 2016). Luminal bladder cancer does not appear to be strongly EMT-dependent seeing that it may cooperate with cancer-associated fibro-blasts (CAFs). Changes in expression concern enrichment of GATA-binding protein 3 (*GATA3*), human epidermal growth factor Receptor 2 (*HER2*), Src kinase or E-Cadherin proteins and mutations of fibroblast growth factor receptor 3 (*FGFR3*), cyclin dependent kinase inhibitor 1A (*CDKN1A*), tuberous sclerosis 1 (*TSC1*) and E74-like ETS transcription factor 3 (*ELF3*) genes (Cancer Genome Atlas Research Network, 2014).

Neuronal bladder cancer

Very few cases (5%) refer to tumors that do not express differentiation biomarkers of either luminal or basal types. They are discriminated by higher proliferation, abundant expression of neuronal and neuroendocrine genes and numerous Tumor Protein p53 (*TP53)* or Retino-blastoma protein 1 (*RB1)* mutations. Com-pletely diverse treatment recommendations go concurrently with different patient survival, being a much worse in that case compared to any luminal or basal variant (Inamura, 2018).

MOLECULAR BACKGROUND OF BLADDER CANCER

A multistep process of carcinogenesis can be divided into three basic stages – initiation, promotion and progression (Said, 2013). Subsequent tumor invasion and epithelial-to-mesenchymal transition which are processes of metastasis are embraced in the final phases of progression, briefly visualized in Figure 1. There are a few groups of factors that can give rise to bladder cancer – environmental and molecular factors or epigenetic and genetic changes within tumor suppressors can be distin-guished (Shin, 2017). In terms of the first group, smoking and exposure to chemical compounds such as aromatic amines, fungicides (Letasiova, 2012), N-nitroso compounds (Catsburg, 2014) or arsenic (Jankovic, 2007) may contribute to the development of bladder cancer, although this is not necessarily the case (Czerniak, 2016). Genetic changes include six critical regions on different chromosomes, in sequence: chromo-some 3 (q22-q24), 5 (q22-q31), 9 (q21-q22), 10 (q26), 13 (q14), and 17 (p13) (Majewski, 2008), with a loss of heterozygosity (LOH) event at the long arm of chromosome 9 considered as one of the very first observed phenomena in bladder cancer (Chow, 2000).

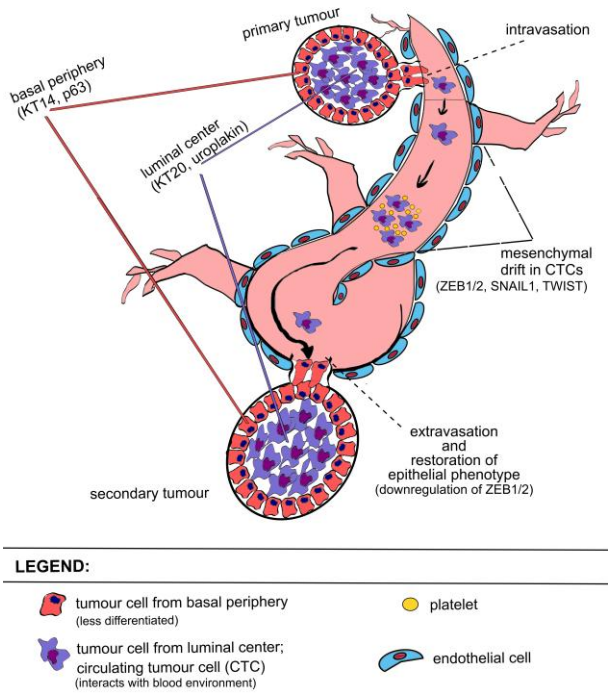

Figure 1. EMT process during tumor expansion (based on (Czerniak, 2016))

Within the epigenetic changes, a group of genes established as forerunners (FRs) plays an important role in the enforcement of field effect which refers to genomic alterations throughout whole bladder mucosa caused by chemical carcinogens (Czerniak, 2016). At least five of such genes have been mapped in the immediate closeness of the *RB1* gene on the thirteenth chromosome in one of the above critical regions – 13q14. This includes G protein-coupled receptor 38 (*GPR38*) which is an alias to motilin receptor (*MLNR*) (McConkey, 2010), lysophosphatidic acid receptor 6 (*LPAR6/P2RY5*), integral membrane protein 2B (*ITM2B*), ADP-ribosylation factor-like protein 11 (*ARL11*), and calcium binding protein 39 like (*CAB39L*) (Czerniak, 2016). The summary of regions' localization is exhibited graphically (Figure 2). FR genes are thought to be novel tumor suppressors that are interrupted during tumor development even before more common anti-oncogenes (McConkey, 2010). Using the example of the *RB1* gene, the forerunners' positioning can take place at a short distance or even within the gene e.g. *CHC1L* (Chromosome condensation 1-like) and *ITM2B* or *P2RY5*, respectively (Majewski, 2008). Using methylation and mutation analysis, Czerniak et al. showed that the FRs are more frequent methylated than they undergo mutations (Czerniak, 2016). Representatives of this group have various functions. For example, *ITM2B* shows similarity to B-cell lymphoma 2 (BCL-2) proteins subfamily containing single BH3 domain (Bcl-2 homology 3 domain), while *P2RY5* is included in G protein-coupled receptors that is responsible for binding lysophosphatidic acid (LPA) – lipid signaling molecule (Fleischer, 2004; Pasternack, 2008). Lee et al. proved that the restoration of the proper functioning of these genes resulted in the induction of tumor cell death via apoptosis and the inhibition of the cell cycle (Lee, 2007). Furthermore, the participation of *LPAR6* and *CAB39L* have been confirmed in the expansion of cells derived from urothelium, which resulted in the development of luminal and basal cancer, respectively (Czerniak, 2016).

Depending on the origin cell that initiates the development of bladder cancer, there are two main pathways of tumor progression – luminal papillary that is characterized by superficiality and basal nonpapillary which leads to direct muscle-invasive manner (McConkey, 2010). These two ways of cancer development correspond to the alleged dual-track concept which was invented in the seventies of the XIX century based on histology and clinical data of bladder (Koss, 1974). At present, the molecular insight separating the two aforementioned pathways has been already carried out.

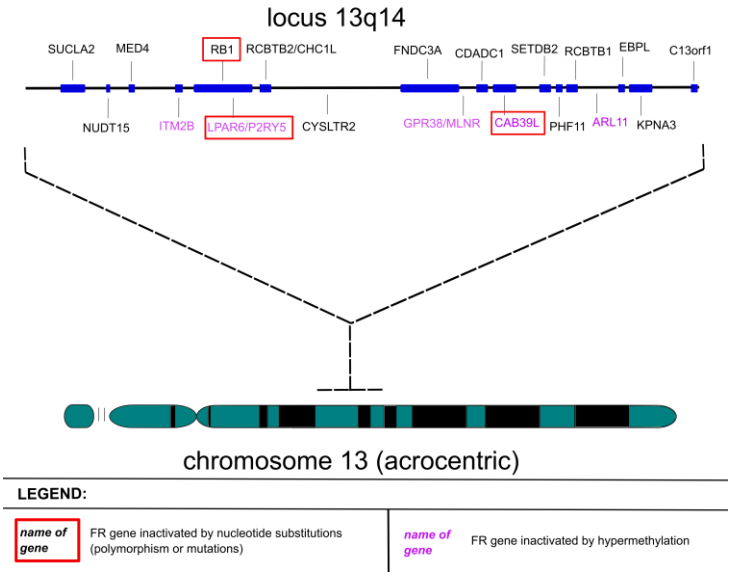

Figure 2. Genomic location of selected forerunner genes (based on (Czerniak, 2016; Majewski, 2008))

Superficial papillary tumor

The onset of this type is associated with hyperplastic changes in urothelium known as low-grade intraurothelial neoplasia (or dysplasia) and concerns 80% of bladder tumor cases. Based on grading they possess low malignancy as this grow with a non-invasive propensity and superficially, thus there is less possibility for metastasis, although 10-15% of tumors could progress into invasive phenotype. Likewise, the phenomenon of relapse is very common in this subtype (Majewski, 2008; McConkey, 2010). As mentioned earlier, various forerunner genes are implicated in diverse subtypes of bladder cancer, therefore *LPAR6* is thought to have an impact on the luminal pathway and is involved in the initiation of field effect which is an inseparable part of urothelial carcinogenesis and drives cell expansion (Czerniak, 2016).

Based on epidemiological studies it is known that chemical induction includes right majority of bladder cancers (at least in the West) (Letasiova, 2012). Triggered inter-mediate uropro-genitor cells were confirmed to be sensitive to one of the nitroso compounds – N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) which could lead to afterward DNA alterations, same as tobacco carcinogens (Czerniak, 2016). One of the modifications is aforementioned allelic loss located in chromosome 9, which concerns inactivation of p16 and IFNα (interferon α) in their loci on 9p21 and 9p22 regions, respectively. As a result, this could lead to disruption of cell cycle regulation or apoptosis which subsequently influence the cancer progression (McConkey, 2010). Proteins associated with luminal papillary carcinoma include forkhead box A1/estrogen receptor 1 (FOXA1/ESR1) interacting pathway, GATA3 or tripartite motif containing 24 (TRIM24) transcription factors. Subsequently formed low-grade papillary tumor may progress to high-grade invasive cancer owing to various structural alterations or copy-number variations (CNVs) (Czerniak, 2016).

Invasive non-papillary tumor

In comparison to the superficial papillary tumor, the solid non-papillary one is derived from high-grade intraurothelial neoplasia (also termed carcinoma in situ – CIS) and principally concerns patients with no previous history. Aggressive invasion of basement membrane and highly metastatic phenotype result in lymph nodes and distant sites occupation (McConkey, 2010). Similar to the above, FR genes are also involved in triggering field effect and cell expansion, however in case of basal uro-progenitor cells the further development can be dependent on *CAB39L* (Czerniak, 2016). Proteins included in pathways that drive progression of basal nonpapillary track are p63, EGFR, signal transducer and activator of transcription 3 (STAT3), or hypoxia-inducible factor 1-alpha (HIF-1α), and what is suggested in other research – participation of PTEN/PI3K/AKT/mTOR pathway (phosphatase and tensin homolog; phosphate-dylinositol-4,5-bisphosphate 3-kinase; serine/threonine kinase 1; mammalian target of rapamycin) is thought to affect muscle-invasive phenotype (Knowles, 2009). Deve-

lopment dependent on PTEN inactivation may also require loss of p53 (or p53 pathway malfunction connected with loss of p21) which promotes in situ carcinoma (Puzio-Kuter, 2009; Stein, 1998). Interestingly, *FGFR3* mutations are found more than twice frequent in superficial papillary tumors, but still occur in invasive nonpapillary cancers in almost 20% (McConkey, 2010). A summary of the development of bladder cancer based on the dual-track concept and depending on the different uniprogenitor cells is presented in figure 3.

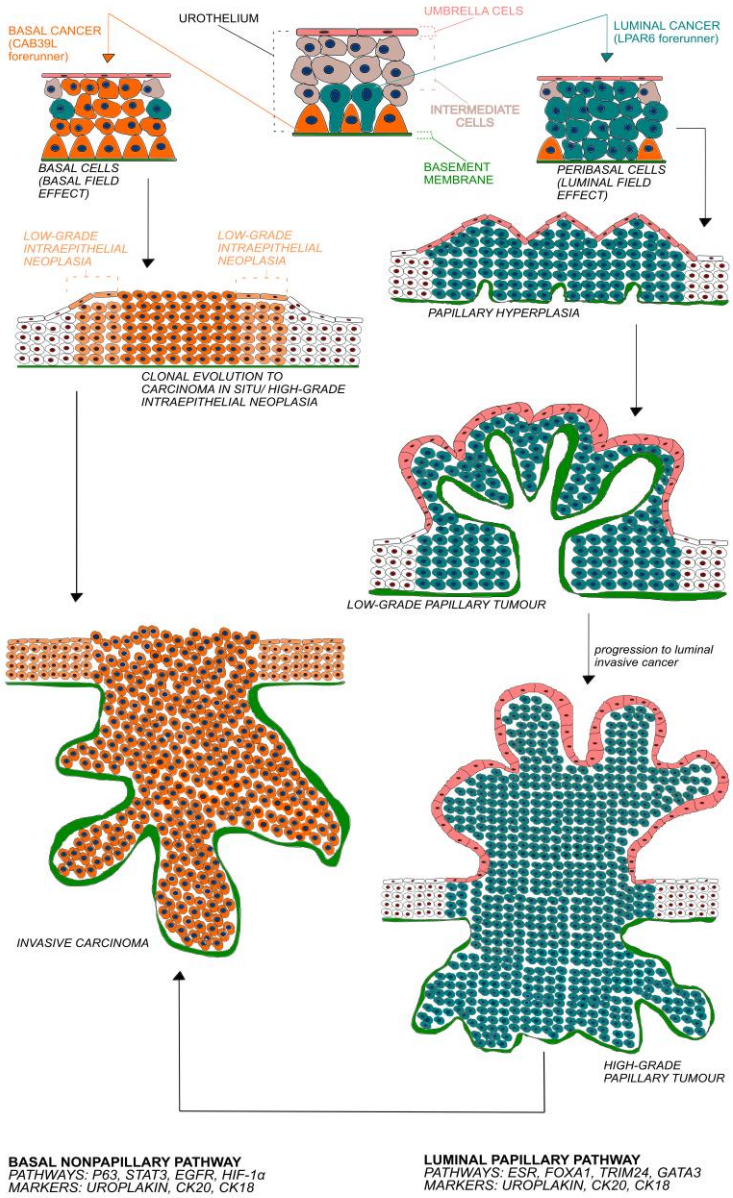

Figure 3. Molecular development of basal and luminal subtypes of bladder cancer (based on (Czerniak, 2016))

TRANSCRIPTION FACTORS (TFS)

Scientific reports indicate that transcription factors shape the expression profile of specific bladder cancer molecular subtype among which examples most frequently given are Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ), Retinoid X Receptor alpha (RXR-α), Forkhead Box M1 (FOXM1), FOXA1, GATA3, and STAT3 (Choi, 2014; Eriksson, 2015; Rebouissou, 2014). Expression of both cell adhesion molecules (CAMs) and matrix metalloproteinases (MMPs) – a groups implycated in steps of tumor invasion – is also thought to be regulated through specific transcription factors. In the case of E-cadherin (representative of CAMs) regulation during cancer development, participation of Snail, Slug

and SMAD Interacting Protein 1 (SIP1) factors were confirmed to suppress its expression (Makrilia, 2009). Secondly, matrix metalloproteinases are known to be regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), STATs, Erythroblast Transformation-Specific (ETS) or Activator Protein 1 (AP-1) and Activating enhancer-binding Protein 2 (AP-2) transcription factor families (Fanjul-Fernandez, 2010; Overall, 2002; Yan, 2007). Representative of the last family of the above – AP-2γ (encoded by *TFAP2C* gene) was assessed as a transcription factor whose overexpression correlates with the distant recurrence or tumor growth (Yamashita, 2019). Such tendency was further supported by our preliminary analysis on the bladder cancer TCGA cohort, which showed a statistically significant relationship where the increase in *TFAP2C* expression worsened prognostic endpoints (disease-free survival (DFS), overall survival (OS)). This prompted us to dwell on this issue in the hope of finding another representative transcription factor whose designation could potentially assist molecular classification.

AP-2 FAMILY OF TRANSCRIPTION FACTORS

Classification of the entire TFs network is based on occurrence of ten superclasses which varies in terms of structural properties and sequence similarity (Ehsani, 2016; Yamashita, 2019). One of the three most-extensive superclasses called Basic Domains contains the AP-2 family which belongs to the basic Helix-Span-Helix (bHSH) class. All genera of bHSH are capable to distinguish particular G/C-rich motifs e.g. CCCCAGGC (Mitchell, 1987), GCCN3/4GGG or GCCN3/4GGC (Mohibullah, 1999). Binding region resembles those of the other classes within Basic Domains superclass, both Basic leucine zipper factors (bZIP) and Basic helix-loop-helix factors (bHLH), whereas domain loop length discriminates bHLH class from bHSH (longer in the second class) (Bolander, 2004). Characteristic regions of bHSH members (thereby AP-2 family factors) are shown in figure 4. N-terminal site of AP-2 members comprises transactivation domain, whilst DNA-binding domain (together with internal dimerization domain (Kannan, 1999)) are located from the C-terminal space (Eckert, 2005). The proper functioning of individual domains is ensured by structural motifs – Proline/Glutamine-rich, basic α-helix and HSH, respectively. The last two, if separated, are still able to link two members of AP-2 family, nonetheless DNA-binding functio-nality is disrupted (Williams, 1991).

This is important because AP-2 factors function as homodimers or heterodimers, consequently the ability to co-interact is essential. All five AP-2 family representatives: α, β, γ, δ and ε (Orlic-Milacic, 2016) are critical for gene expression regulation along with apoptosis or cell cycle control during proper early developmental stages (Hilger-Eversheim, 2000). Initial location of AP-2 factors is nucleus and their activity can be modulated by regulation of subcellular localization (Pellikainen, 2004), DNA-binding capability (Mazina, 2001), transactivation potential (Aqeilan, 2004) or degradation (Li, 2006) – those are possible through post-translational phosphorylation (Garcia, 1999), sumoylation (Zhong, 2003), reduction/oxidation reactions (Huang, 1998), or via interactions with other proteins (Eckert, 2005). Despite the participation of whole family in normal development, their overexpression has already been observed in distinct tumors (Hoei-Hansen, 2004; Jager, 2005; Pellikainen, 2004).

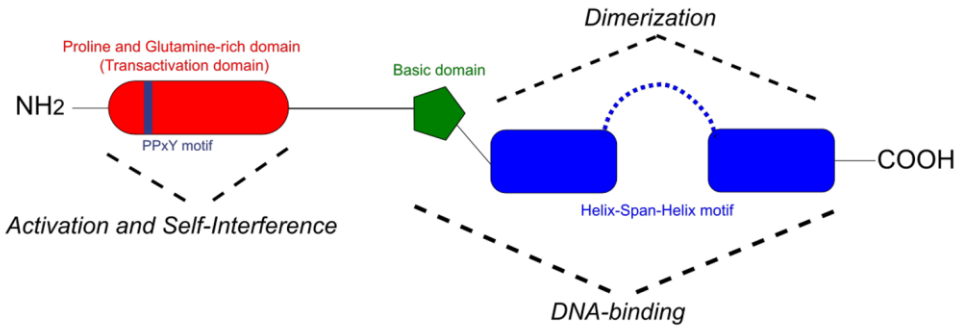

Figure 4. Specific domains of AP-2 family (based on (Eckert, 2005; Kannan, 1999; LiCalsi, 2000))

TFAP2C gene

Gene encoding AP-2γ is localized on the twentieth chromosome (cytological location: 20q13.31) on the plus strand and its overall size is 9,982 of bases with seven exons within. This AP-2 family member encodes transcription factor that recognizes specific sequence SCCTSRGGS (S = G/C, R = A/G) (Woodfield, 2010) and is implicated in the development of eyes, limbs, face or neural tube through genes activation. In accordance with the NCBI Reference Sequence Database, only one mRNA variant undergoes transcription from this gene (accession number: NM_003222.3). Graphical presentation of *TFAP2C* gene and additional information are presented in figure 5.

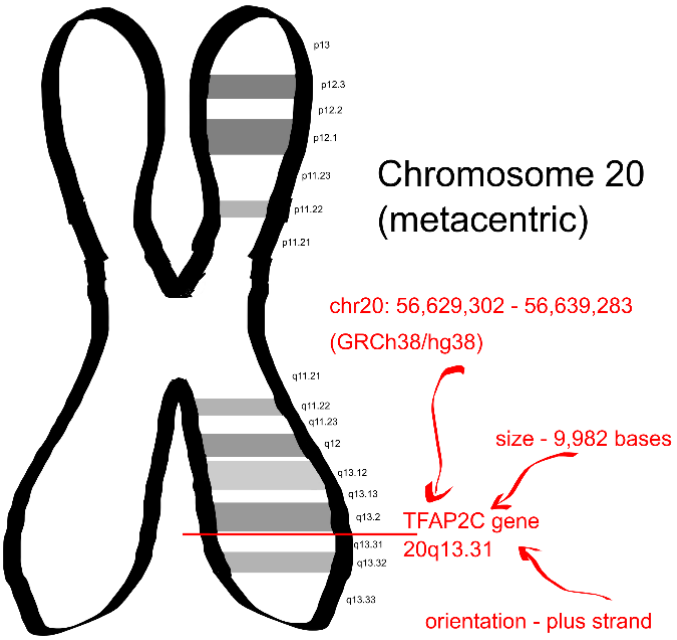

Figure 5. Localization of TFAP2C gene (based on GeneCards database)

AP-2γ protein

Properties of AP-2γ protein e.g. dimerization, sequence recognition or influence on cellular and viral enhancers – all resembles entire AP-2 family characteristics described above. UniProt KnowledgeBase determines that there is only one isoform in addition to the canonical sequence (identifiers: Q92754-2 and Q92754-1, respectively). AP-2γ activity can be modulated via post-translational modifications (PTMs) however only one location is experimentally acknowledged and has their cause-and-effect relation-ship confirmed i.e. sumoylation at lysine 10 that leads to inhibition of activity (Eloranta, 2002). An interesting issue is a way AP-2γ functionality is considered – predominantly it is perceived as oncogene yet some cases are suggesting its tumor suppressor capa-bilities (Kolat, 2019). Still, literature data predomi-nantly indicate the oncogenic nature of this factor, which confirms the reliability of our preliminary analyzes. The behavior of this factor has been reliably studied on the example of interaction with WW Domain Containing Oxidoreductase (*WWOX*) suppressor, during which AP-2γ proliferation-promoting activity is inhibited (Aqeilan, 2004).

Despite the enormity of available knowledge describing affected signaling pathways such as FGFR3/RAS, TP53/RB1 or PI3K/AKT/mTOR, the heterogeneity of bladder cancer inclines a more thorough understanding of this cancer by identifying potentially useful genes. By initial insight of databases, we noticed significant differences e.g. in the level of *TFAP2C* in normal tissue compared to cancerous or the effect of different expression of this gene on the survival of oncological patients. Therefore, we aimed our research to perform *in silico* analysis for determination of AP-2γ role in bladder cancer.

MATERIALS AND METHODS

GEPIA (Gene Expression Profiling Interactive Analysis, gepia2.cancer-pku.cn) was used for analyzing prognostic endpoints on the BLCA cohort depending on *TFAP2C* level.

UALCAN (http://ualcan.path.uab.edu) was used to analyze the expression level of *TFAP2C* gene, its prognostic value (according to median values of genes) and correlation with clinic-pathological parameters. This portal performs analyses of cancer OMICS data (TCGA and MET500) (Chandrashekar, 2017).

cBioPortal for Cancer Genomics (https://www.cbioportal.org) was applied to analyze CNVs of *TFAP2C* and correlation with another genes.

TCGA (http://cancergenome.nih.gov) was used to extract clinical data of 412 BLCA cases (status of May 2, 2020) together with their mRNAseq profiling (level 3 RNASeqV2, RSEM normalized). For further analyses, we excluded non-tumor type of samples (according to TCGA Barcode, acquired from https://docs.gdc.cancer.gov) which resulted in total data of 408 patients.

GSEA (Gene Set Enrichment Analysis, http://genepattern.broadinstitute.org/gp) was conducted using entire database of molecular signatures (eight major collections acquired from http://software.broadinstitute.org/gsea/msigdb/index.jsp). 5175 genes being targets for AP-2γ were taken into account for enrichment analysis. Targets for transcription factor were combined using three databases (excluding duplicates): Gene Transcription Regulation Database (GTRD, version 19.10 (Yevshin, 2019; Yevshin, 2017)), TRANScription FACtor database (TRANSFAC, version 2019.2) and Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRRUST, version v2). Defined phenotypes ("TFAP2C high" or "TFAP2C low") were established by the median value of AP-2γ and restricted to extreme patients to show larger differences between groups. Functional analysis was performed using the tTest metric with a weighted statistic to score hits/misses and permutation type concerning phenotype.

DAVID (Database for Annotation, Visualization and Integrated Discovery) Bio-informatics Resources (Huang, 2009) (version 6.8, https://david.ncifcrf.gov) were used to annotate genes to processes they regulate, their molecular function, localization or implicated signaling pathways. All annotation databases were included during analyses.

RESULTS

DIFFERENCES OF GENE EXPRESSION DEPENDING ON TISSUE TYPE, TUMOR STAGE, HISTOLOGY, MOLECULAR SUBTYPES, NODAL METASTASIS AND MUTATION STATUS

To understand the role of AP-2γ transcription factor, at the beginning we compared their mRNA level in cancer tissue and normal tissue in patients using UALCAN tool. We observed statistically significant higher expression of *TFAP2C* in cancer compared to normal bladder tissue (fig. 6A). We also analyzed how the level of its expression changed depending on clinical data. We observed increasement of *TFAP2C* expression in II-IV bladder cancer stages (with the highest noticed in stage IV) compared to normal tissue (fig. 6B). Moreover, higher mRNA level of *TFAP2C* was connected with mutation in *TP53* gene in bladder cancer (fig. 6C). The analyses of *TFAP2C* expression level in different histological and molecular subtypes also shows some significant relationships compared to normal tissue (fig. 6D and E). Finally, mRNA level of *TFAP2C* increases during metastasis to lymph nodes (fig. 6F).

DEPENDENCE OF PROGNOSTIC ENDPOINTS ON THE LEVEL OF *TFAP2C* GENE

To investigate the connection between *TFAP2C* expression and survival prognosis, we used Kaplan-Meier plots form GEPIA repository. Using median cutoff of overall group and 95% confidence interval, we observed that high *TFAP2C* expression correlates with unfavorable prognosis i.e. DFS and OS (HR = 1.6, $p = 0.0069$; HR = 1.4, $p = 0.024$, respectively) (fig. 7).

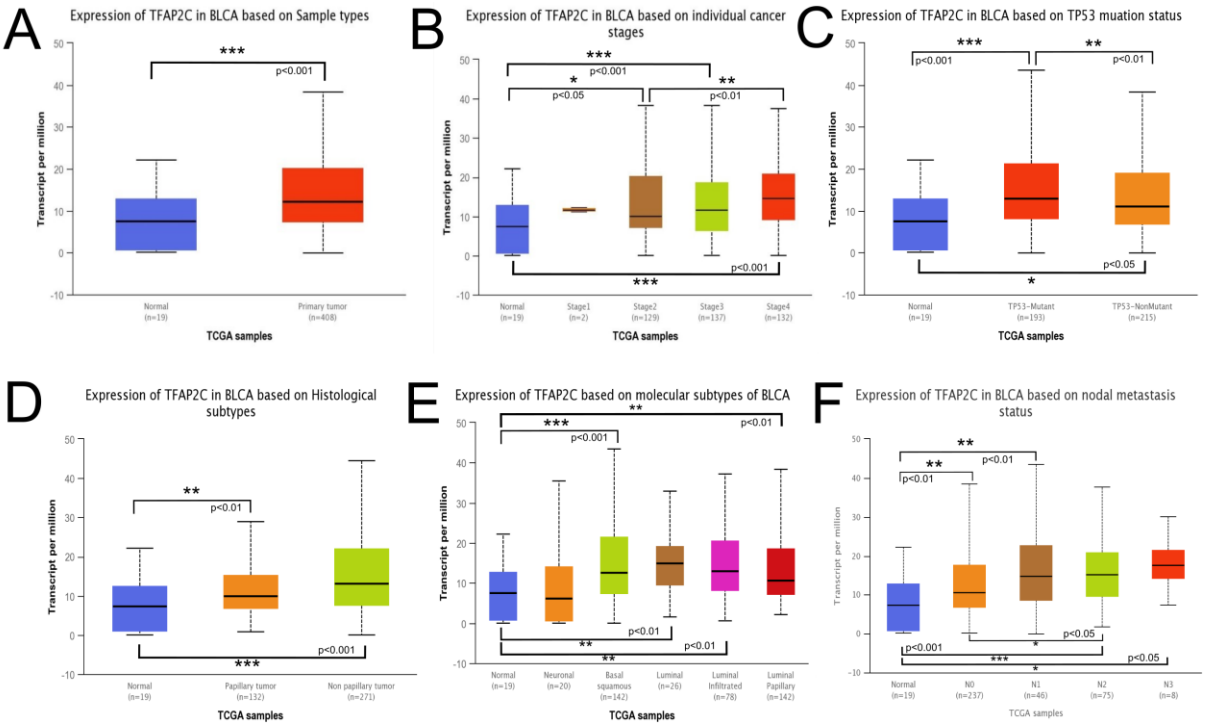

Figure 6. Variability of TFAP2C mRNA expression levels for selected UALCAN characteristics.
(A) Sample/tissue type. (B) Cancer stages. (C) TP53 mutation status. (D) Histological subtypes.
(E) Molecular subtypes. (F) Nodal metastasis status

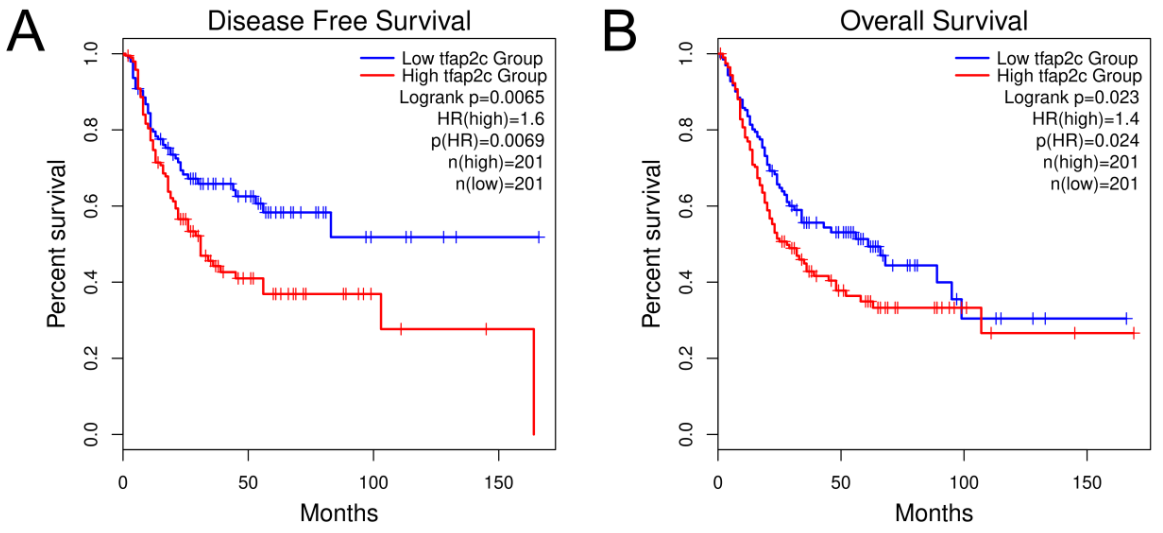

Figure 7. Prognostic endpoints analysis according to mRNA expression of TFAP2C gene.
(A) Disease-Free Survival. (B) Overall Survival

COPY-NUMBER VARIATIONS AND CO-EXPRESSION ANALYSIS

Subsequently, we examined whether the changes in *TFAP2C* expression may result from copy number alternations using bladder cancer patients' data (TCGA, Firehose Legacy) via cBioPortal database (fig. 8).

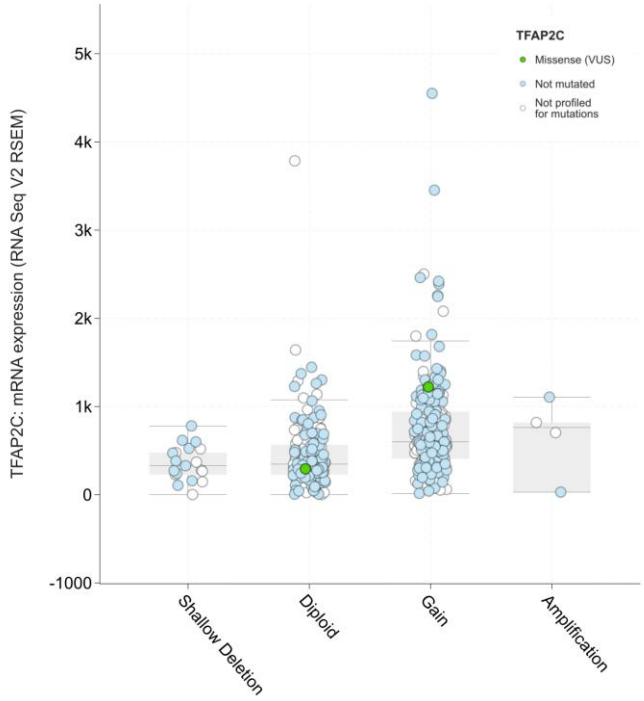

Figure 8. Putative copy-number alterations of TFAP2C gene in the BLCA cohort

Mutations were observed only in "diploid" and "gain" of *TFAP2C* gene. There was not noticed gene amplification, so we can conclude that changes in *TFAP2C* expression in bladder cancer are not due to the copy number alternations.

Using RNAseq of 413 BLCA patients from cBioPortal (Z-score threshold = 0) we aimed to identify genes correlating with *TFAP2C* expression and the processes they are implicated in. Co-expression analysis showed that *TFAP2C* positively correlates with 99 genes (Spearman's rank correlation coefficient ≥ +0.3) and negatively with 19 genes (Spearman's rank correlation coefficient ≤ -0.3). Ontological classification of selected genes was presented in figure 9.

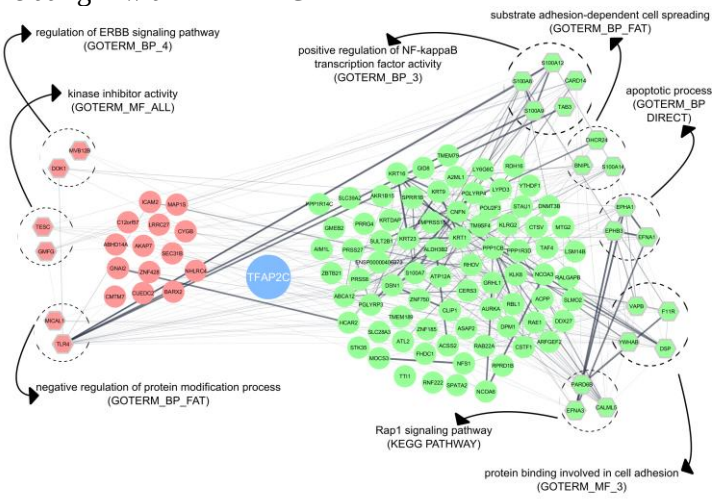

Figure 9. Genes negatively (red) and positively (green) co-expressed with TFAP2C according to cBioPortal with additional selected ontology classification

GENE SET ENRICHMENT ANALYSIS

Important gene sets established through GSEA concerned biological signatures of BIOCARTA canonical pathways, chemical & genetic perturbations (CGP), gene ontology (GO): biological processes (BP), hallmarks or regulatory target gene sets. Collectively, 10 gene sets enriched in "TFAP2C high" were significant at FDR < 0.25 while 3 gene sets in "TFAP2C low" significant at p < 0.01. Exemplary heatmaps for both phenotypes are presented in Figure 10.

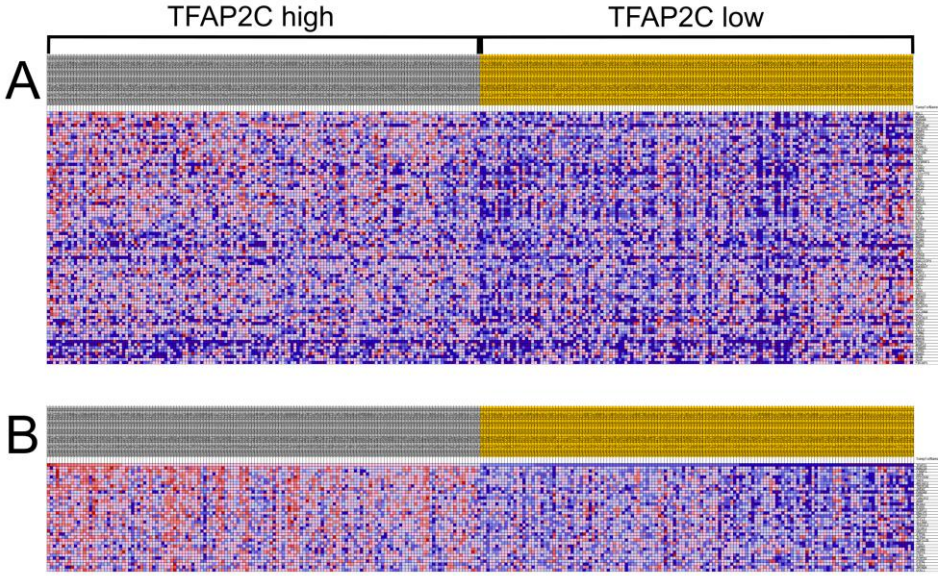

Figure 10. Heatmaps generated from GSEA analysis (only core enrichment genes). (A) LINDGREN_BLADDER_CANCER_CLUSTER_3_UP gene set. (B) NIKOLSKY_ BREAST_CANCER_20Q12_Q13_AMPLICON gene set

FUNCTIONAL ANNOTATION CLUSTERING

Afterwards, each gene set (only core enrichment genes) was implemented into DAVID for further data mining. Detailed subdivision and obtained gene-annotation enrichments from GSEA gene sets can be found in table 1.

Table 1. Gene-annotation enrichment analysis of selected gene sets. Gene sets were chosen with the highest possible significance for each phenotype (FDR < 0.25 for "TFAP2C high"; p < 0.01 for "TFAP2C low")

Phenotype	MSigDB collection	Selected gene set	Selected annotation database	Description
TFAP2C high	C2:CGP	NIKOLSKY_BREAST_CANCER_20Q12_Q13_AMPLICON	GO:BP_DIRECT	Regulation of cell proliferation
		LINDGREN_BLADDER_CANCER_CLUSTER_3_UP	KEGG	PI3K-Akt signaling pathway
		HOLLERN_EMT_BREAST_TUMOR_DN	GO:BP_DIRECT	Negative regulation of cell-cell adhesion
		LANDIS_BREAST_CANCER_PROGRESSION_UP	GO:BP_ALL	Positive regulation of cellular process
		WHITFIELD_CELL_CYCLE_M_G1	GO:BP_DIRECT	Intermediate filament organization

		OUELLET_OVARIAN_CANCER_INVASIVE_VS_LMP_UP	KEGG	Hippo signaling pathway
		BIDUS_METASTASIS_UP	KEGG	MAPK signaling pathway
	C2:CP Reactome	REACTOME_TRANSCRIP-TIONAL REGULATION_BY_THE_AP_2_TFAP2_FAMILY_OF_TRANS-CRIPTION_FACTORS	GO:BP_DIRECT	Negative regulation of apoptotic process
	C2:BP Biocarta	BIOCARTA_HER2_PATHWAY	KEGG	ERBB signaling pathway
	Hallmarks	HALLMARK_G2M_CHECKPOINT	GO:BP_DIRECT	G2/M transition of mitotic cell cycle
TFAP2C low	C3:MIR	MIR4265	KEGG	Transcriptional misregulation in cancer
		MIR4296	GO:BP_DIRECT	Cell differentiation
		MIR4713_3P	GO:BP_ALL	Positive regulation of cell communication

DISCUSSION

BLCA is a tumor whose frequency has decreased in recent years. The classification takes into account the division into histological or molecular subtypes, with the latter considering e.g. categorization by the transcription factors which may indicate not only the expression profile but also the potential treatment type resulting from the available actionable targets. Because the identification of both new molecules involved in signaling pathways and TFs guiding their expression is crucial, we focused on investigating the transcription factor whose upregulation significantly worsened the prognostic endpoints of bladder cancer patients. Using available literature, there was a noticeable trend indicating the oncogenic nature of *TFAP2C* in various cancers. While the role of AP2γ-encoding gene in lung cancer was ambiguous (Chang, 2017; Kang, 2017; Kim, 2016), the data for breast cancer (Gee, 2009; Williams, 2009), testicular carcinoma (Hoei-Hansen, 2004), primary ovarian tumors (Odegaard, 2006), melanoma (Lal, 2013) or neuroblastoma (Gao, 2014) are consistent indicating association of *TFAP2C* with e.g. increased proliferation, tumor growth, cell cycle progression but also treatment resistance or poorer prognosis. Analysis of the putative copy-number alteration in relevant cohorts using cBioPortal (TCGA Firehose Legacy studies for breast, testis, ovary, skin; Broad, Nature 2015 for neuroblastoma) indicated that the observed *TFAP2C* effect on the above processes and prognosis is not due to gene mutations. The same conclusions could be drawn from the BLCA analysis (TCGA Firehose Legacy study), which encouraged the selection of most strongly co-expressed genes (correlation less than -0.3 or more than +0.3) that resulted in the extraction of 118 genes further analyzed ontologically. Within such, additional gene ontology indicated regulation of pathways (ERBB, Rap1), apoptosis or adhesion-dependent cell spreading. With the use of UALCAN web resource we performed expression analysis in terms of BLCA characteristics such as cancer stages, nodal metastasis, *TP53* mutation status and histological subtypes or molecular subtypes, collectively indicating that *TFAP2C* expression increases with stage or metastasis status and possesses highest level in luminal subtype (molecularly) or non-papillary subtype (histologically). Additionally, its expression is higher in tumors with positive p53 mutational status.

Lastly, there was statistically significant difference between *TFAP2C* expression in normal tissue compared to cancerous (in favor of the latter). To understand what molecular alterations occur with different levels of *TFAP2C* in bladder cancer patients, a gene set enrichment analysis was performed on the BLCA cohort subdivided into groups with high or low *TFAP2C* expression through the context of AP-2γ targets. Collected data included gene sets related to canonical pathways, genetic perturbations, biological processes, hallmarks or regulatory target gene sets. Subsequently, functional annotation enrichment analysis was performed on genes that have contributed the most. Considering the group with high level of *TFAP2C* gene expression, contribution to numerous signaling pathways (MAPK, ERBB, Hippo, PI3K/Akt) regulation was proposed along with e.g. negative impact on apoptotic process or cellular adhesion. Contrastingly, in "TFAP2C low" group the enriched gene sets were associated with regulatory targets guided by microRNA (miRNA) which has a reference to the literature because many interactions of AP-2 family members with long non-coding RNA (lncRNA) or miRNA have been proven (Kolat, 2019). Nevertheless, gene ontology of the most significant gene sets for "low" phenotype revealed functional annotation connected with positive cell communication or cell differentiation, which indicates the alleged one absolute difference between the "high" and "low" groups associated with intercellular communication – in the first group, intercellular commu-nication is supposedly limited by loss of adhesion, while in the second group the increase in intercellular communication was noted. We speculate that such state may result from different levels of *TFAP2C*, which in the case of a decrease in its expression directly impacts on miRNA network and allows the regulation of processes that are not possible in the case of *TFAP2C* high expression.

SHORT CONCLUSION

To conclude, computational analysis of AP-2γ role showed that "TFAP2C high" group is more associated with phenomena or signaling pathways that are perceived negatively when excessively occur at molecular level. Instead, results for the "TFAP2C low" group indicate the regulation of cellular processes via a wide range of miRNA transcripts, which concurs with the literature data of the present yet minorly explored network of interaction between AP-2γ and miRNA or lncRNA molecules. We believe that further research towards profound exploration of regulated processes not only on the AP2-RNA axis but with investigating other regulators of the protective phenotype, will allow a deeper understanding of the scenario that occur with low *TFAP2C* expression in bladder cancer patients.

REFERENCES

Aine M., Eriksson P., Liedberg F., Hoglund M., Sjodahl G., **On molecular classification of bladder cancer: Out of one, many**, In: Eur Urol. 2015; 68(6):921-3.

American Cancer Society, **Cancer Statistics Center,** In: American Cancer Society, 2020; Accessed 21 May 2020, http://cancerstatisticscenter.cancer.org/.

Aqeilan R.I., Palamarchuk A., Weigel R.J., Herrero J.J., Pekarsky Y., Croce C.M., **Physical and functional interactions between the wwox tumor suppressor protein and the ap-2gamma transcription factor**, In: Cancer Res. 2004; 64(22):8256-61.

Bolander F.F., **Molecular Endocrinology 3rd Edition**, 2004; In: Academic Press; ISBN: 9780121112325.

Cancer Genome Atlas Research Network, **Comprehensive molecular characterization of urothelial bladder carcinoma,** In: Nature, 2014; 507(7492):315-22.

Cassell A., Yunusa B., Jalloh M., Mbodji M.M., Diallo A., Ndoye M., et al., **Non-muscle invasive bladder cancer: A review of the current trend in africa**, In: World J Oncol. 2019; 10(3):123-131.

Catsburg C.E., Gago-Dominguez M., Yuan J.M., Castelao J.E., Cortessis V.K., Pike M.C., et al., **Dietary sources of n-nitroso compounds and bladder cancer risk: Findings from the los angeles bladder cancer study**, In: Int J Cancer, 2014; 134(1):125-35.

Chandrashekar D. S., Bashel B., Balasubramanya S. A. H., Creighton C. J., Ponce-Rodriguez I., Chakravarthi B., et al. **Ualcan: A portal for facilitating tumor subgroup gene expression and survival analyses**. In: Neoplasia, 2017; 19(8):649-658.

Chang T.H., Tsai M.F., Gow C.H., Wu S.G., Liu Y.N., Chang Y.L., et al., **Upregulation of microrna-137 expression by slug promotes tumor invasion and metastasis of non-small cell lung cancer cells through suppression of tfap2c**, In: Cancer Lett. 2017; 402:190-202.

Choi W., Porten S., Kim S., Willis D., Plimack E.R., Hoffman-Censits J., et al., **Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy**, In: Cancer Cell, 2014; 25(2):152-65.

Chow N.H., Cairns P., Eisenberger C.F., Schoenberg M.P., Taylor D.C., Epstein J.I., et al., **Papillary urothelial hyperplasia is a clonal precursor to papillary transitional cell bladder cancer**, In: Int J Cancer. 2000; 89(6):514-8.

Czerniak B., Dinney C., McConkey D., **Origins of bladder cancer**, In: Annu Rev Pathol. 2016; 11:149-74.

Dadhania V., Zhang M., Zhang L., Bondaruk J., Majewski T., Siefker-Radtke A., et al., **Meta-analysis of the luminal and basal subtypes of bladder cancer and the identification of signature immunohistochemical markers for clinical use**, In: EBioMedicine, 2016; 12:105-117.

Eckert D., Buhl S., Weber S., Jager R., Schorle H., **The ap-2 family of transcription factors**, In: Genome Biol. 2005; 6(13):246.

Ehsani R., Bahrami S., Drablos F., **Feature-based classification of human transcription factors into hypothetical sub-classes related to regulatory function**, In: BMC Bioinformatics, 2016; 17(1):459.

Eloranta J.J., Hurst H.C., **Transcription factor ap-2 interacts with the sumo-conjugating enzyme ubc9 and is sumolated *in vivo***, In: J Biol Chem. 2002; 277(34):30798-804.

Eriksson P., Aine M., Veerla S., Liedberg F., Sjodahl G.Hoglund M., **Molecular subtypes of urothelial carcinoma are defined by specific gene regulatory systems**, In: BMC Med Genomics, 2015; 8:25.

Fanjul-Fernandez M., Folgueras A.R., Cabrera S., Lopez-Otin C., **Matrix metalloproteinases: Evolution, gene regulation and functional analysis in mouse models**, In: Biochim Biophys Acta, 2010; 1803(1):3-19.

Fleischer A., Rebollo A., **Induction of p53-independent apoptosis by the bh3-only protein itm2bs**, In: FEBS Lett. 2004; 557(1-3):283-7.

Gao S.L., Wang L.Z., Liu H.Y., Liu D.L., Xie L.M., Zhang Z.W., **Mir-200a inhibits tumor proliferation by targeting ap-2gamma in neuroblastoma cells**, In: Asian Pac J Cancer Prev. 2014; 15(11):4671-6.

Garcia M.A., Campillos M., Marina A., Valdivieso F., Vazquez J., **Transcription factor ap-2 activity is modulated by protein kinase a-mediated phosphorylation**, In: FEBS Lett. 1999; 444(1):27-31.

Gee J.M., Eloranta J.J., Ibbitt J.C., Robertson J.F., Ellis I.O., Williams T., et al., **Overexpression of tfap2c in invasive breast cancer correlates with a poorer response to anti-hormone therapy and reduced patient survival**, In: J Pathol. 2009; 217(1):32-41.

Hilger-Eversheim K., Moser M., Schorle H., Buettner R., **Regulatory roles of ap-2 transcription factors in vertebrate development, apoptosis and cell-cycle control**, In: Gene. 2000; 260(1-2):1-12.

Hoei-Hansen C.E., Nielsen J.E., Almstrup K., Sonne S.B., Graem N., Skakkebaek N.E., et al., **Transcription factor ap-2gamma is a developmentally regulated marker of testicular carcinoma in situ and germ cell tumors**, In: Clin Cancer Res. 2004; 10(24):8521-30.

Huang da W., Sherman B.T., Lempicki R.A., **Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists**, In: Nucleic Acids Res. 2009; 37(1):1-13.

Huang Y., Domann F.E., **Redox modulation of ap-2 DNA binding activity *in vitro***, In: Biochem Biophys Res Commun. 1998; 249(2):307-12.

Inamura K., **Bladder cancer: New insights into its molecular pathology**, In: Cancers (Basel). 2018; 10(4).

Jager R., Friedrichs N., Heim I., Buttner R., Schorle H., **Dual role of ap-2gamma in erbb-2-induced mammary tumorigenesis**, In: Breast Cancer Res Treat. 2005; 90(3):273-80.

Jankovic S., Radosavljevic V., **Risk factors for bladder cancer**, In: Tumori. 2007; 93(1):4-12.

Kang J., Kim W., Lee S., Kwon D., Chun J., Son B., et al., **Tfap2c promotes lung tumorigenesis and aggressiveness through mir-183- and mir-33a-mediated cell cycle regulation**, In: Oncogene. 2017; 36(11):1585-1596.

Kannan P., Tainsky M.A., **Coactivator pc4 mediates ap-2 transcriptional activity and suppresses ras-induced transformation dependent on ap-2 transcriptional interference**, In: Mol Cell Biol. 1999; 19(1):899-908.

Kim W., Kim E., Lee S., Kim D., Chun J., Park K.H., et al., **Tfap2c-mediated upregulation of tgfbr1 promotes lung tumorigenesis and epithelial-mesenchymal transition**, In: Exp Mol Med. 2016; 48(11):e273.

Knowles M.A., Platt F.M., Ross R.L., Hurst C.D., **Phosphatidylinositol 3-kinase (pi3k) pathway activation in bladder cancer**, In: Cancer Metastasis Rev. 2009; 28(3-4):305-16.

Kolat D., Kaluzinska Z., Bednarek A.K., Pluciennik E., **The biological characteristics of transcription factors ap-2alpha and ap-2gamma and their importance in various types of cancers**, In: Biosci Rep. 2019; 39(3).

Koss L.G., Tiamson E.M., Robbins M.A., **Mapping cancerous and precancerous bladder changes. A study of the urothelium in ten surgically removed bladders**, In: JAMA. 1974; 227(3):281-6.

Lal G., Contreras P.G., Kulak M., Woodfield G., Bair T., Domann F.E., et al., **Human melanoma cells over-express extracellular matrix 1 (ecm1) which is regulated by tfap2c**, In: PLoS One. 2013; 8(9):e73953.

Lee S., Jeong J., Majewski T., Scherer S.E., Kim M.S., Tuziak T., et al., **Forerunner genes contiguous to rb1 contribute to the development of in situ neoplasia**, In: Proc Natl Acad Sci U S A. 2007; 104(34):13732-7.

Letasiova S., Medve'ova A., Sovcikova A., Dusinska M., Volkovova K., Mosoiu C., et al., **Bladder cancer, a review of the environmental risk factors**, In: Environ Health. 2012; 11 Suppl 1:S11.

Li M., Wang Y., Hung M.C., Kannan P., **Inefficient proteasomal-degradation pathway stabilizes ap-2alpha and activates her-2/neu gene in breast cancer,**. In: Int J Cancer, 2006; 118(4):802-11.

LiCalsi C., Christophe S., Steger D.J., Buescher M., Fischer W.Mellon P.L., **Ap-2 family members regulate basal and camp-induced expression of human chorionic gonadotropin**, In: Nucleic Acids Res. 2000; 28(4):1036-43.

Majewski T., Lee S., Jeong J., Yoon D.S., Kram A., Kim M.S., et al., **Understanding the development of human bladder cancer by using a whole-organ genomic mapping strategy,** In: Lab Invest. 2008; 88(7):694-721.

Makrilia N., Kollias A., Manolopoulos L., Syrigos K., **Cell adhesion molecules: Role and clinical significance in cancer**, In: Cancer Invest. 2009; 27(10):1023-37.

Mazina O.M., Phillips M.A., Williams T., Vines C.A., Cherr G.N., Rice R.H., **Redistribution of transcription factor ap-2alpha in differentiating cultured human epidermal cells**, In: J Invest Dermatol. 2001; 117(4):864-70.

McConkey D.J., Choi W., Ochoa A., Dinney C.P.N., **Intrinsic subtypes and bladder cancer metastasis**, In: Asian J Urol. 2016a; 3(4):260-267.

McConkey D.J., Choi W., Shen Y., Lee I.L., Porten S., Matin S.F., et al., **A prognostic gene expression signature in the molecular classification of chemotherapy-naive urothelial cancer is predictive of clinical outcomes from neoadjuvant chemotherapy: A phase 2 trial of dose-dense methotrexate, vinblastine, doxorubicin, and cisplatin with bevacizumab in urothelial cancer,**. In: Eur Urol. 2016b; 69(5):855-62.

McConkey D.J., Lee S., Choi W., Tran M., Majewski T., Lee S., et al., **Molecular genetics of bladder cancer: Emerging mechanisms of tumor initiation and progression**, In: Urol Oncol. 2010; 28(4):429-40.

Mitchell P.J., Wang C., Tjian R., **Positive and negative regulation of transcription in vitro: Enhancer-binding protein ap-2 is inhibited by sv40 t antigen**, In: Cell. 1987; 50(6):847-61.

Mohibullah N., Donner A., Ippolito J.A., Williams T., **Selex and missing phosphate contact analyses reveal flexibility within the ap-2[alpha] protein: DNA binding complex**, In: Nucleic Acids Res. 1999; 27(13):2760-9.

Odegaard E., Staff A.C., Kaern J., Florenes V.A., Kopolovic J., Trope C.G., et al., **The ap-2gamma transcription factor is upregulated in advanced-stage ovarian carcinoma**, In: Gynecol Oncol. 2006; 100(3):462-8.

Orlic-Milacic M., **Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors Current neurology and neuroscience reports**, In: U.S. National Library of Medicine, 2016; Accessed 21 May 2020 <https://www.ncbi.nlm.nih.gov/biosystems/1427839/>.

Overall C.M., Lopez-Otin C., **Strategies for mmp inhibition in cancer: Innovations for the post-trial era**, In: Nat Rev Cancer. 2002; 2(9):657-72.

Pasternack S.M., von Kugelgen I., Al Aboud K., Lee Y.A., Ruschendorf F., Voss K., et al., **G protein-coupled receptor p2y5 and its ligand lpa are involved in maintenance of human hair growth**, In: Nat Genet. 2008; 40(3):329-34.

Pellikainen J., Naukkarinen A., Ropponen K., Rummukainen J., Kataja V., Kellokoski J., et al., **Expression of her2 and its association with ap-2 in breast cancer**, In: Eur J Cancer. 2004; 40(10):1485-95.

Puzio-Kuter A.M., Castillo-Martin M., Kinkade C.W., Wang X., Shen T.H., Matos T., et al., **Inactivation of p53 and pten promotes invasive bladder cancer**, In: Genes Dev. 2009; 23(6):675-80.

Rebouissou S., Bernard-Pierrot I., de Reynies A., Lepage M.L., Krucker C., Chapeaublanc E., et al., **Egfr as a potential therapeutic target for a subset of muscle-invasive bladder cancers presenting a basal-like phenotype**, In: Sci Transl Med. 2014; 6(244):244ra91.

Said N., Frierson H.F., Sanchez-Carbayo M., Brekken R.A., Theodorescu D.,**Loss of sparc in bladder cancer enhances carcinogenesis and progression**, In: J Clin Invest. 2013; 123(2):751-66.

Shin J.H., Lim J.S., Jeon B.H., **Chapter 3 - Pathophysiology of Bladder Cancer** In: Ku Hyeon J. **Bladder Cancer**, 2017; In: Academic Press; ISBN: 0-12809-940-2.

Stein J.P., Ginsberg D.A., Grossfeld G.D., Chatterjee S.J., Esrig D., Dickinson M.G., et al., **Effect of p21waf1/cip1 expression on tumor progression in bladder cancer**, In: J Natl Cancer Inst. 1998; 90(14):1072-9.

Williams C.M., Scibetta A.G., Friedrich J.K., Canosa M., Berlato C., Moss C.H., et al., **Ap-2gamma promotes proliferation in breast tumour cells by direct repression of the cdkn1a gene**, In: EMBO J. 2009; 28(22):3591-601.

Williams T., Tjian R., **Characterization of a dimerization motif in ap-2 and its function in heterologous DNA-binding proteins**, In: Science. 1991; 251(4997):1067-71.

Wojciechowska U., Didkowska J., **Zachorowania i zgony na nowotwory złośliwe w Polsce,** In: Krajowy Rejestr Nowotworów, 2017; Accessed 21 May 2020, http://onkologia.org.pl/.

Woodfield G.W., Chen Y., Bair T.B., Domann F.E., Weigel R.J., **Identification of primary gene targets of tfap2c in hormone responsive breast carcinoma cells**, In: Genes Chromosomes Cancer, 2010; 49(10):948-62.

Yamashita H., Kawasawa Y.I., Shuman L., Zheng Z., Tran T., Walter V., et al., **Repression of transcription factor ap-2 alpha by ppargamma reveals a novel transcriptional circuit in basal-squamous bladder cancer**, In: Oncogenesis. 2019; 8(12):69.

Yan C., Boyd D.D., **Regulation of matrix metalloproteinase gene expression**, In: J Cell Physiol. 2007; 211(1):19-26.

Yevshin I., Sharipov R., Kolmykov S., Kondrakhin Y., Kolpakov F., **Gtrd: A database on gene transcription regulation-2019 update**, In: Nucleic Acids Res. 2019; 47(D1):D100-D105.

Yevshin I., Sharipov R., Valeev T., Kel A., Kolpakov F., **Gtrd: A database of transcription factor binding sites identified by chip-seq experiments**, In: Nucleic Acids Res. 2017; 45(D1):D61-D67.

Zhong L., Wang Y., Kannan P., Tainsky M.A., **Functional characterization of the interacting domains of the positive coactivator pc4 with the transcription factor ap-2alpha**, In: Gene. 2003; 320:155-64.