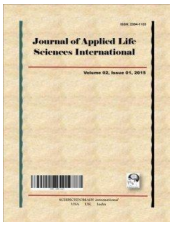

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Uncovering the Selective Drug Targets in Urethane-Mediated Lung Cancer through Network Approach

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Authors' contributions

This work was carried out in collaboration between all authors. Authors THF and SD designed the study, author ASO provided relevant literature information and author THF performed the computational analysis and wrote the first draft of the manuscript. All authors revised the draft manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To identify the driver targets associated with urethane mediated tumorigenesis by pharmacokinetics prediction, target prediction and gene expression network analysis.
Methodology: Standard bioinformatics tools were used, which include SwissADME, SwissTargetPrediction, eXpression2Kinases (X2K), and ClustalO.
Results: It was found that urethane has very low lipophilicity and high gastrointestinal absorption. Urethane major probable targets include tyrosyl-DNA phosphodiesterase 1 (TDP1), acetyl cholinesterase and muscarinic acetylcholine receptors. Enrichment analysis showed that transcription factors most expressed through urethane-targeted genes include TRIM28, RELA, SUZ12 and EGR1 while protein-protein interaction analysis showed that these transcription factors were mostly coordinated by heat shock protein 90 (Hsp90) isoforms (HSP90AA1, HSPAB1 and HSP90B1). The implicated targets were highly associated with cyclin-dependent kinases (CDKs) and mitogen-activated protein kinases (MAPKs).

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Conclusion: Selective inhibition of TDP1 and Hsp90 isoforms and not transcription factors, could be the central therapeutic point for suppression and prevention of lung tumour.

Keywords: Cancer; tumorigenesis; urethane; ethyl carbamate; TDP1; Hsp90; gene network.

1. INTRODUCTION

The phenomena origin of cancer has been reduced to the physics and chemistry quantitation of cellular processes while the ambiguous use of mutation and carcinogenic agents unless metabolically defined, could hinder better understanding and prevention of cancer [1]. Cancer is a leading cause of death globally and approximately 15 million new cancer cases will be diagnosed as the world population reached 7.5 billion by 2020 [2]. It has been reported that the cancer cells originate from normal body cells in two sequential phases. First is the irreversible injuring of respiration while the second phase involves a struggle for adaptation or survival by the injured cells to maintain the native structure during which cell paralysis occur in some parts due to lack of energy while another part succeeded in replacing the demised respiration energy by fermentation energy [1]. Due to the differential pathway for fermentation from that of respiration, the highly differentiated body cells are then converted into undifferentiated cells which grow malignantly [1]. Cancer cells utilize multiple strategies such as high glycolytic flux, redox signalling and modulation of autophagy to avoid cell death and overcome nutritional deficiency [3].

Cancer is caused by both internal factors such as inherited mutations, hormones, and immune conditions; and environmental/acquired factors such as tobacco, diet, radiation, and infectious organisms [4]. Among the most important modifiable risk factors for cancer are tobacco use; overweight, obesity and physical inactivity; harmful alcohol use; infections; air pollution (outdoor and indoor) and occupational carcinogens [5,6]. Missense mutations in the human gene such as RAS genes namely Kirsten rat sarcoma viral oncogene homolog (KRAS), neuroblastoma rat sarcoma viral oncogene homolog (NRAS) and Harvey rat sarcoma viral oncogene homolog (HRAS), have been implicated as one of the drivers of tumor initiation and maintenance, where they function as GDP–GTP-regulated binary on-off switches, which regulate cytoplasmic signaling networks that control diverse normal cellular processes [7].

Urethane (ethyl carbamate), an ester of carbamic acid, has been found mainly as a by-product of fermented foods and beverages such as spirit, wine, beer, bread, soy sauce, and yoghurt [8,9,10]. Urethane could be made by the reaction of ethanol and urea or by warming urea nitrate with ethanol and sodium nitrite [11] as well as via addition of ethanol to cyanate which could be an explanation for the high concentration of urethane in stone-fruit spirits [12,13]. It has been formed from substances like citrulline and N-carbamyl compounds during foods and beverage fermentations [8]. The presence of readily substituted side group in the basic structure of urethane ($ROC(O)NH_2$), lead to the formation of polymer with possible diverse functional groups in addition to or exclusion of urethane group [14]. Urethane has been used, for the manufacture of meprobamate - a tranquillizer drug; as a crease-resistant finish in the textile industry; as a solvent, in hair conditioners, in the preparation of sulfamic acids; as an extractant of hydrocarbons from crude oil and as a food flavour-enhancing agent [15,16]. Urethane is widely used in veterinary medicine as an anesthetic for laboratory animals where it functioned as multitarget compound on neurotransmitter-gated ion channels in its mechanism of action [17]. Urethane has been reported to have an effect on polyp diseases, hematopoietic system and peripheral blood cells with pathologic changes in lung, kidney, female gonad and liver, in experimental animals and man [18-20].

However, in human health, urethane has been described as a multi-site carcinogen [16]. The urethane model of lung tumorigenesis has been used; to identify genetic modifiers of lung cancer risk, to unravel the role of KRAS in tumour progression [21,22], and to research chemoprevention, tumour biology and early detection of cancer [23,24]. The irreversible inhibition of cell respiration by urethane has been reported and that the inhibition is more irreversible at the higher temperature [1]. Urethane typically induces lung tumours by increasing IL-1β processing in neutrophils with NF-κB inhibition [25,26]. Study has implicated the non-canonical NF-κB component p52 in urethane-induced lung carcinogenesis and

suggest modulation of p52 activity as new therapeutic targets [27].

Multiple genomic studies substantiated the notion of cancer as an evolutionary process that can readily adapt within the lifetime of a patient but it has remained a major challenge to use genomic information to make accurate predictions for individual cancer patients [28]. It is evident that most biological discovery will come from the complex interaction of all the proteins and cells working with environmental factors, not driven directly by the genetic code [4]. The aim of this study is to investigate novel targets which are responsible for urethane-mediated carcinogenesis. Cancer is a systems-level disease [29] and to find cancer-specific drug targets, a systems-level context using network approach is inevitable.

2. MATERIALS AND METHODS

2.1 *In silico* Preparation of ligand and Pharmacokinetics Prediction

The chemical structure of the ligand (urethane) was obtained from the PubChem Compound Database (http://www.ncbi.nlm.nih.gov/pccompound) in canonical SMILES (Simplified Molecular Input Line Entry Specification) format. The *in silico* ADME (Absorption, Distribution, Metabolism, and Excretion) of the ligands were carried out using SwissADME server (http://www.swissadme.ch) and ADME screening was performed at default parameters [30].

2.2 Genes Target Prediction

The identification of potential target genes for urethane was carried out using the SwissTargetPrediction server (http://www.swisstargetprediction.ch) and *Homo sapiens* was selected as the target organism [31]. Cancer Brower of COSMIC database (GRCh38 · COSMIC v85) (https://cancer.sanger.ac.uk/cosmic) [32], was searched using tissue selection (Lung), sub-tissue selection (Include all), histological selection (Include all) and sub-histological selection (Bronchioloalveolar adenocarcinoma). The genes associated with bronchioloalveolar adenocarcinoma (newly called adenocarcinoma-in-situ [33,34]) were obtained and compared with urethane target genes.

2.3 Target Gene Expression Analyses

The upstream regulatory networks from signatures of differentially expressed genes obtained from urethane target prediction were determined by transcription factor enrichment analysis, protein-protein interaction network expression and kinase enrichment analysis, using eXpression2Kinases (X2K) Web server (http://amp.pharm.mssm.edu/X2K/#g2n) [35].

2.4 Phylogenetic Analysis

The sequence of ten transcription factors obtained from gene expression analysis was extracted from UniprotKB/Swiss-prot database (www.uniprot.org). Multiple sequence alignment was carried out using ClustalO (https://www.ebi.ac.uk/Tools/msa/clustalo), at the default setting, and phylogenetic tree was constructed. The real phylogeny was visualized at http://phylo.io using tree data obtained from ClustalO [36].

3. RESULTS AND DISCUSSION

Cancer is a disease involving dynamic changes in the genome, and the genomes of tumour cells are altered at multiple sites in many ways, as subtle as point mutations and as obvious as chromosome rearrangement [37]. The summary of the predicted ADME of urethane as shown in Table 1 indicates that it has very low lipophilicity (XlogP) with high gastrointestinal absorption (GA) and higher potential of being synthesised from organic sources due to high synthetic accessibility score (S). The study has shown that for a drug-like compound, $5 \leq$ lipophilicity $\geq 0 \leq$ hydrophilicity ≥ -5 [38]. The synthetic accessibility score (S) ranges between 1 (easy to make) and 10 (very difficult to make) while partition coefficient (LogP) and solubility coefficient (LogS) are parameters used to evaluate the bioavailability score (BS) [38]. Higher GA of urethane possibly underscores its major route of xenobiotic which is predominantly from food source. High skin permeation also underscores the urethane potential to diffuse through the membrane of the lung cells when inhaled. The ADME showed that urethane has no inhibitory effect on cytochrome P450 1A2, 2C19, 2C9, 2D6 and 3A4. However, studies have shown that cytochrome P450 2E1 (CYP2E1) is involved in about 96% of urethane digestion to carbon dioxide in wild-type mice [39,40].

As shown in Table 2, inducible nitric oxide synthase and cyclin-dependent kinases have been reported for urethane mediated cancer [41]. However, urethane major probable targets include tyrosyl-DNA phosphodiesterase 1 (TDP1), acetyl cholinesterase, butyryl cholinesterase, and muscarinic acetylcholine receptors. These targets have been implicated in Alzheimer's disease, cancer and chronic cough respectively [38,42-44]. The involvement of TDP1 as a key target for cancer treatments has been established in numerous studies and yet to be successfully targeted with the inhibitor for approved use as anticancer drug in the market [43,45-47].

In order to meet the required standard for expression analysis on X2K server, the following genes; phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic alpha (PIK3CA), epidermal growth factor receptor (EGFR), Kirsten rat sarcoma (KRAS), insulin growth factor (IGF1) and vascular endothelial growth factor (VEGF) were added to the urethane predicted target genes, based on their involvement in cancer as reported in the literatures [8,41,48]. Differential gene expression analysis has become one of the key approaches to identify genes important in the diagnosis and prediction of cancer progression. Gene expression profiles have been used for the reverse engineering of cancer specific regulatory networks [49,50].

From the large number of tumour-associated genetic changes only a few plays a key role in tumour pathogenesis (called driver mutations). Driver mutations can be characterised by their pathway association. In many tumours, p53, Ras and PI3K are the major signalling pathways containing driver mutations [7,27,51]. Genes with co-occurring mutations in the COSMIC database prefer direct signalling interactions. The top 20 genes obtained from COSMIC database for adenocarcinoma-in-situ as shown in Fig. 1, were: EGFR, KRAS, TP53, CDKN2A, LRP1B, STK11, NCOR1, FCRL4, MED12, ARID1B, BRCA1, KMT2C, SMAD4, ATRX, NF1, DDX3X, NOTCH2, DEK, PBRM1 and ERBB2.

The protein-protein interaction in Fig. 2 showed transcription factors most expressed by urethane-targeted genes based on hypergeometric p-value which include TRIM28 (transcription intermediary factor 1-beta), RELA/TF56 (Rel-like domain-containing protein A or transcription factor p65), KAT2A (histone acetyltransferase), NFE2L2 (nuclear factor erythroid 2-related factor 2), CBX3/TF1B (chromobox protein homolog 3), SUZ12 (polycomb protein), EGR1 (early growth response protein 1), GATA2 (endothelial transcription factor 2), FOXM1 (forkhead box protein M1) and ZMIZ1 (zinc finger MIZ domain-containing protein 1). These transcription factors have been implicated in one or several biological processes which include positive gene regulation, tumorigenesis, apoptosis, induction chromatin remodelling of the proviral gene, up-regulation of genes in response to oxidative stress, regulation of cellular redox conditions, epigenetic repression systems and other molecular functions. For example, TRIM28 is a multidomain protein with versatile functions in transcription and deoxyribonucleotide repair and overexpressed in many epithelial cancers such as breast, lung, ovarian, liver, gastric and colorectal tumours [52,3].

The transcription factor enrichment analysis results in Fig. 3 indicated the best fifteen transcription factor. Based on the network analysis, KAT2A targets HSP90AB1 and HSP90B1, FOXM1 targets HSP90B1 and CDK1; TRM28 targets HSP90AB1 and NOS3; NFE2L2 targets PIK3CA, IGF1, KRAS and CHRM3; EGR1 targets EGFR and NOS1; GATA2 targets ACHE, PIK3CA and NOS3; ZMIZI targets TDP1, HSP90AB1 and HSP90AA1; CBX3 targets NOS3; RELA targets HSP90B1 and HSP90AB1; SUZ12 targets CHRM2; IRF8 targets HSP90AB1; MYC targets HSP90AB1 and HSP90AA1; ZC3H11A targets ACHE; NANOG targets HSP90AA1 and IGF1; and FOS targets TDP1 and HSP90B1. This result showed the involvement of heat-shock protein 90 (Hsp90) isoforms in lung tumorigenesis and corroborate previous studies [37,53]. Hsp90 association with fibroblast growth factor (FGF) has been reported [54] whereas FGF signalling network has been implicated in tumour development [55,56]. Mutations in KRAS gene has been shown to promotes malignant pleural effusion in patients with metastatic breast or lung cancer; a condition where excess fluid is build-up in the pleural cavity [57]. Malignant pleural effusion and ascites by cancer cells could be an outcome of cellular fermentation instead of cellular respiration in normal cells.

Fifteen kinases with highest hypergeometric p-value found in association with urethane target genes transcription factors include cyclin-dependent kinases (CDKs) and mitogen-activated protein kinases (MAPKs) as shown in

Fig. 4, while combined urethane-mediated gene expression networks is presented in Fig. 5. The involvement of CDKs is required for replication of many lethal viruses such as human papillomaviruses (HPV), human immunodeficiency virus type 1 (HIV-1), human cytomegalovirus (HCMV) and herpes simplex virus (HSV) [36] where these viruses have been implicated as a potent causative agent of cancer. For example, sexually transmitted HPV was found to be the leading risk factor for cervical cancer in women in low-and-middle-income countries [5].

Hsp90 is an essential partner for many signaling protein kinases that are required for efficient cell growth and proliferation. The activity and the stability of the kinases such as cell surface receptor kinases, Src family tyrosine kinases, Raf family protein kinases, MAPK-related protein kinases, cyclin-dependent kinases, casein kinase II (CK2) etc., are dependent on the molecular chaperone activity of Hsp90 [37]. Therefore, inhibition of Hsp90 could be the central therapeutic point for lungs tumour suppression.

Curated data on UniProt/Swiss-Prot showed that ERG1, RELA, ZMIZ1, TRIM28 and SUZ12 are involved in protein sumoylation pathway while ERG1, RELA, CBX3, GATA2, TRIM28, SUZ12 and KAT2A are involved in histone acetylation/methylation function. The phylogeny

Table 1. Predicted pharmacokinetics parameters of urethane

Ligand Name and SMILES	Pharmacokinetics Parameters				
	MW	**HA**	**AH**	**FC**	**RB**
Urethane	89.09 g/mol	6	0	0.67	2
	HBA	**HBD**	**MR**	**TPSA**	**XlogP**
CCOC(=O)N	2	1	20.94	52.32 A	-0.15
	LogS	**GA**	**BBB**	**P-GP**	**CYP**
	-0.17	High	No	No	No
	Log K_p	**LiV**	**BS**	**LeV**	**SA**
	-6.95 cm/s	0	0.55	1	1.10

Physicochemical properties: Molecular weight (MW), Heavy atom (HA), Aromatic heavy atoms (AH), Fraction Csp3 (FC), Rotatable bonds (RB), H-bond acceptors (HBA), H-bond donors (HBD), Molar Refractivity (MR), Total polar surface area (TPSA). Lipophilicity: XLOGP3. Water Solubility: ESOL Log S. Pharmacokinetics: GI absorption (GA), Blood-brain barrier (BBB), P-glycoprotein substrate (P-GP), Cytochrome P450 inhibitor 1A2, 2C19, 2C9, 2D6, 3A4 (CYP), Skin permeation (Log K_p). Druglikeness: Lipinski violations (LiV), Bioavailability Score (BS), Medicinal Chemistry: Leadlikeness Violation (LeV), Synthetic accessibility (SA).

Table 2. Predicted targets of urethane

Predicted targets	Gene common name	UniProt ID	Urethane
Tyrosyl-DNA phosphodiesterase 1	TDP1	Q9NUW8	****
Muscarinic acetylcholine receptor M1, M2, M3, M4	CHRM1, CHRM2, CHRM3, CHRM4,	P11229, P08172, P20309, P08173	***
Acetylcholinesterase	ACHE	P22303	***
Cholinesterase (by homology)	BCHE	P06276	***
Heat shock protein HSP 90-alpha	HSP90AA1	P07900	**
Heat shock protein HSP 90-beta (by homology)	HSP90AB1	P08238	**
Endoplasmin (by homology)	HSP90B1	P14625	**
Nitric oxide synthase, endothelial	NOS3	P29474	**
Nitric oxide synthase, brain	NOS1	P29475	**
Nitric oxide synthase, inducible	NOS2	P35228	**
Cyclin-dependent kinase 2	CDK2	P24941	*
Cyclin-dependent kinase 1 (by homology)	CDK1	P06493	*

**20-25%, **25-30%, ***30-35%, ****35-40% Probability on Target. Probabilities have been computed based on a cross-validation. They may therefore not represent the actual probability of success for any new molecule.*

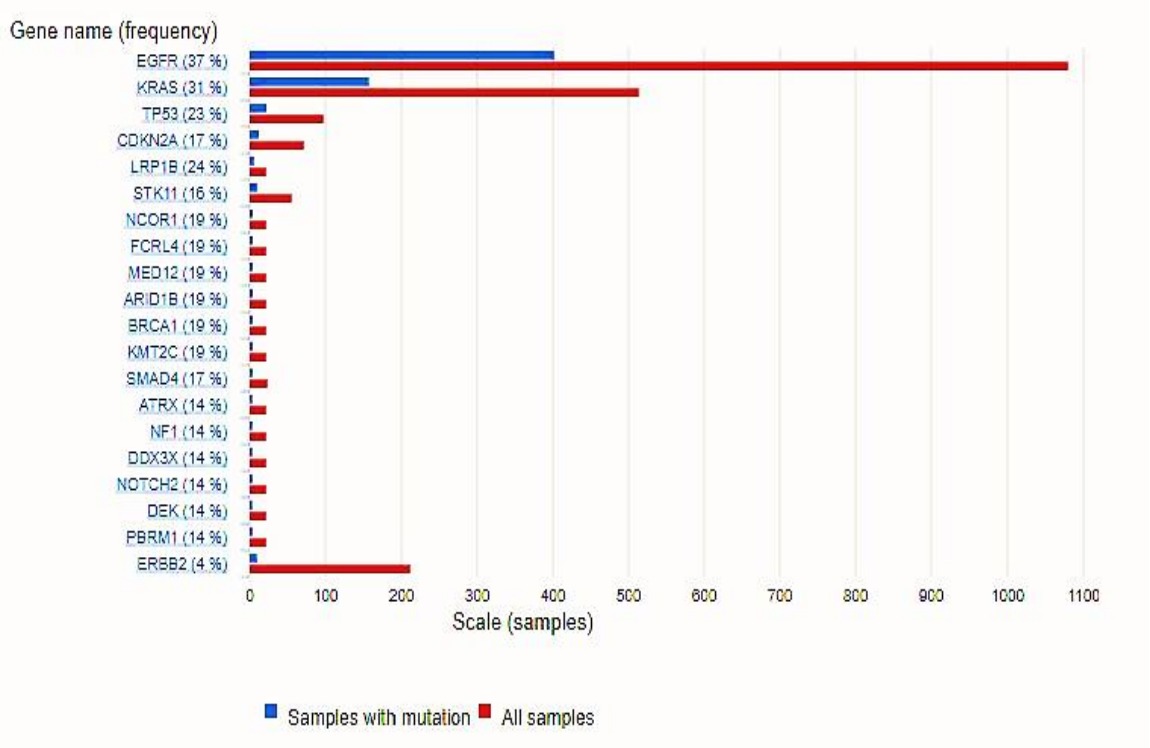

Fig. 1. Top 20 genes associated with Adenocarcinoma-in-situ

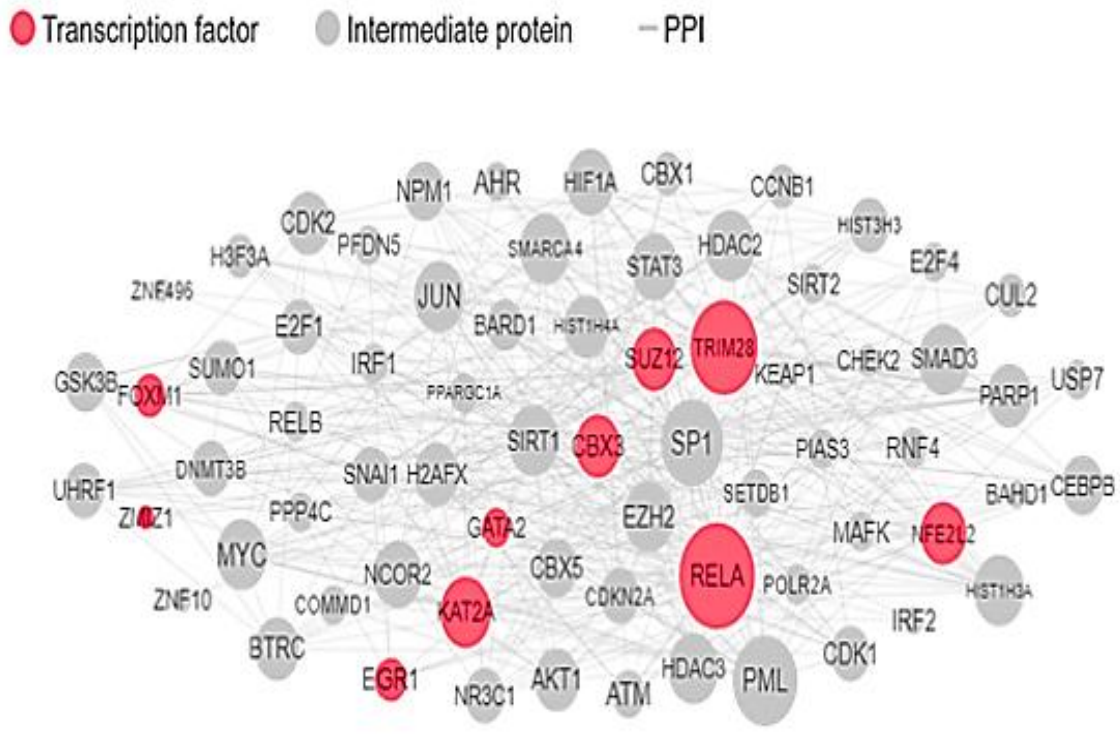

Fig. 2. Protein-protein interaction network expression for urethane target genes

of urethane-mediated gene expression transcription factors as shown in Fig. 6, indicates evolutionary divergences which makes transcription factors to be redundant targets in cancer therapy. Tumour prevention or suppression is not feasible if the research focuses on the transcription factors.

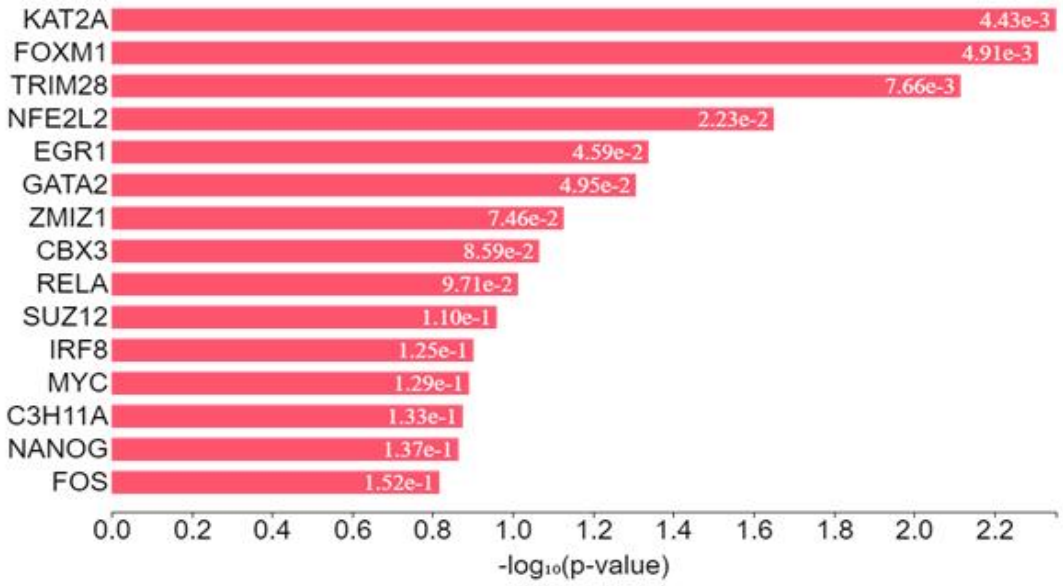

Fig. 3. Transcription factor enrichment analysis for urethane target genes

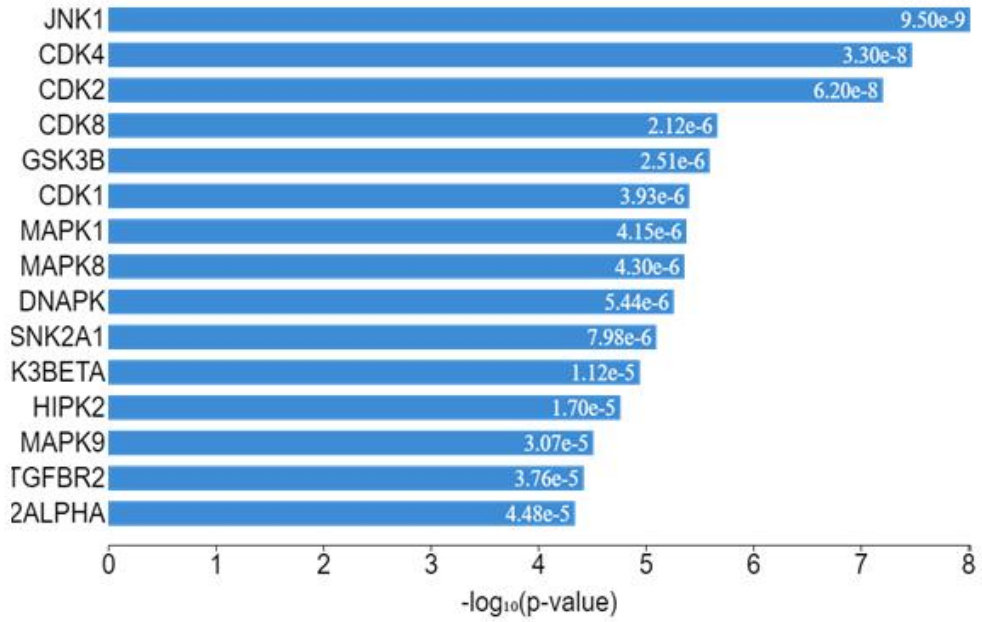

Fig. 4. Kinase enrichment analysis for urethane target genes

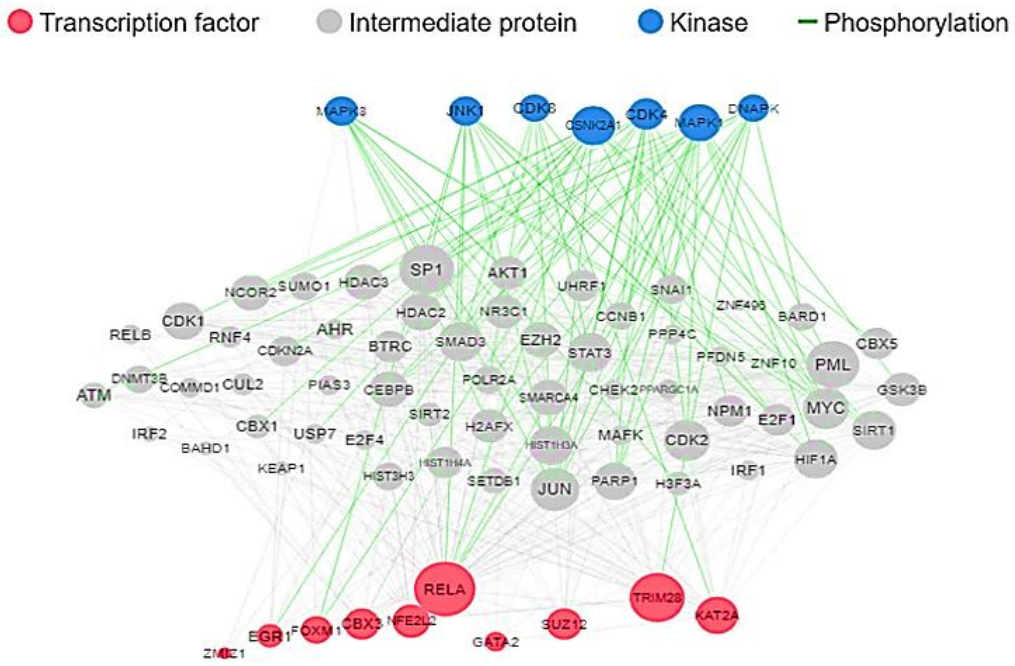

Fig. 5. Overall eXpression2Kinases for urethane target genes

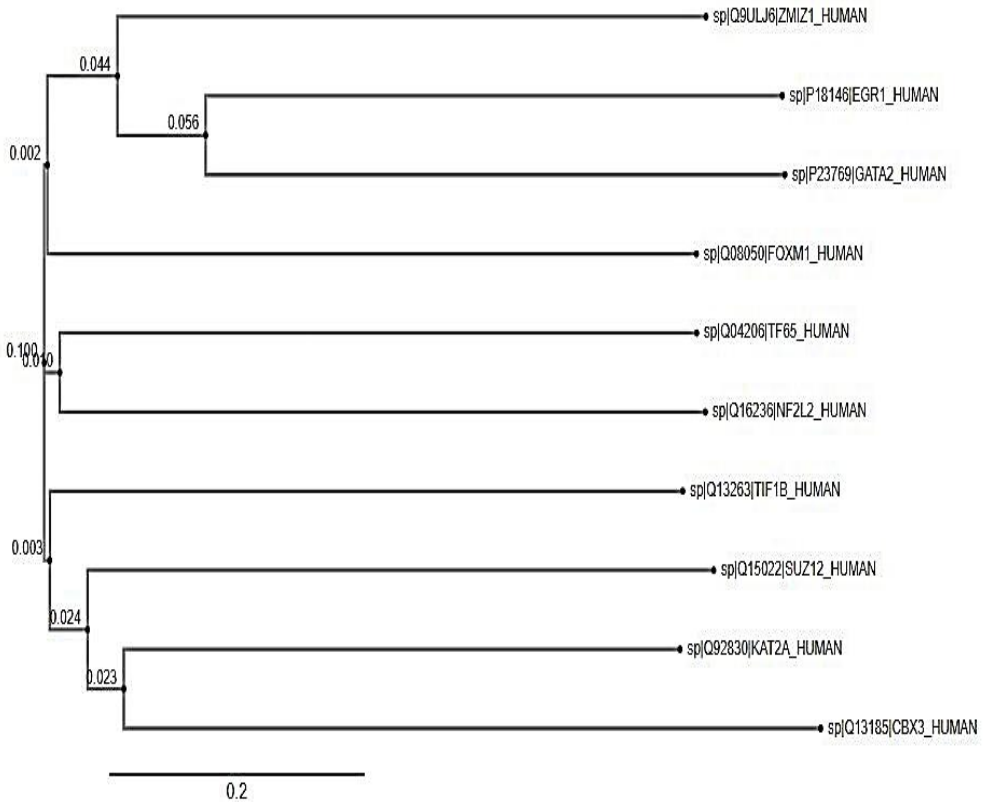

Fig. 6. Phylogeny of urethane-mediated gene expression transcription factors. The numbers indicate the branch length from the closest node, calculated by neighbor-joining method.

4. CONCLUSION

This study suggested the involvement of TDP1 and Hsp90 isoforms as the targets associated with urethane mediated lung tumorigenesis. Further clinical researches are needed to validate the mechanism by which the proliferation of cancerous cells occurs through these two proteins. Translational research for the development of the potent selective inhibitors and suppressors of TDP1 and Hsp90 respectively and multitarget inhibition of the implicated kinases (MAPKs and CDKs), could lead to dramatic reduction in the death cases associated with cancers. Overall, subjects requiring investigation should be cogent studies of arrays of existing substrates/inhibitors in literatures for specificities and kinetic as well as intrinsic thermodynamic parameter of Hsp90, TDP1 and implicated kinases and the impact of oxidative stress on these targets in relevance to cancer pathophysiology and therapy, because there are few works available on these aspects. These will direct what classes of inhibitors should be optimised to enhance the development of potent therapeutants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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