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Evaluation of Cyclin-Dependent Kinase Inhibitor Signalling network in esophageal Adenocarcinoma Via computational and statistical approaches

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Cyclin-dependent kinase inhibitor is a tumor suppressor gene which play essential role in cell cycle. Pervious work demonstrates that, mutation in *CDKN2A* gene cause uncontrolled proliferation, genomic stability, oncogenesis, and metastasis. To evaluate the role of *CDKN2A* signalling network in Esophageal adenocarcinoma we utilized cluster profiler, STRING database and cbioportal. Hypergeometric test was applied within R platform for the detection of biological function of *CDKN2A* and its interactor's genes in the form of enrichmap including cnetplot and Dot plot. Possibility of four different oncogenic mutations (X153- splice/D153N) were observed on amino acid number 153 of *CDKN2A* genes among various samples. We reported that, *TP53* is also highly mutated gene in Esophageal cancer, 87% cases associated with 180 *TP53* mutations. In majority of cases, 15 R248Q/W mutations was observed at P53-DNA binding domain (95-288) of *TP53*. During network analysis, a solid association between *CDKN2A* and *TP53* was observed while co-occurrence between *CDKN2A* and *TP53* was detected in Esophageal adenocarcinoma during mutual exclusivity analysis (Log Odds Ratio= 1.521, p-value= 0.024, q-value=0.270). Our findings indicates that *CDK6*, *MYC*, *CCND1*, *TP53* along with *CDKN2A* are very essential targets for multi-targeted therapy against Esophageal adenocarcinoma. Our research provide a new avenue to explore genomic features of *CDKN2A* and *TP53* in Esophageal adenocarcinoma.

Keywords: *CDKN2A;* Esophageal Adenocarcinoma; Mutations; Networks; Interactions; Targets; Genomic.

INTRODUCTION

Esophageal cancer (EC) is the eighth major prevalent cancer in the world while the sixth most-common death related diseases globally (Ferlay et al. 2013). Esophageal cancer is divided into two major categories that is: 1) Esophageal adenocarcinoma (EAC), 2) esophageal squamous cell carcinoma (ESCC). Geographical distribution show that esophageal adenocarcinoma is most prevalent in the western nations like Europe and North America, although ESCC is common in the people of south-eastern and central Asia, predominantly China and Japan (Arnold et al. 2015). In 2005, 49,000 new cases of esophageal

cancer were reported worldwide. According to global report the prevalence of other cancers was decreased for upcoming 10 decade whereas the incidence of esophageal cancer is increased by 140% up to 2025. In 2005, approximately 40, 0000 patients died due to poor prognosis of this disease, and its rate is increasing day by day (Lambert and Hainaut 2007). Only in china, approximately 477900 esophageal cancer patients diagnosed while in 375000 death cases were reported in 2015 (Chen et al. 2016).

A tumor suppressor gene '*CDKN2A'* abbreviated as; cyclin-dependent kinase inhibitor 2A which is located on chromosome number 9p2 (Nakashima et al. 1999) and play important role at G1/S phase of cell cycle including slowing cell cycle progressing. Many cyclin-dependent kinases were also inhibiting by *CDKN2A* (Serrano et al. 1993; Kamb, Gruis et al. 1994). Inactivation of *CDKN2A* gene due to hyper methylation in promoter region is previously described by different scientists in many cancers like lung (Tam et al. 2013), head and neck(Pierini et al. 2014), hepatocellular (Csepregi et al. 2010), breast (Sinha et al. 2008), and esophageal cancers (Ito, Ohga et al. 2007).

Here, we performed computational and statistical analysis to generate the network of *CDKN2A* and its interactor's genes that are associated with esophageal adenocarcinoma. Furthermore, we evaluated the signaling network of *CDKN2A* gene, oncoprint analysis and mutational analysis via computational tools and databases. Survival analysis was also performed via bioinformatics approach. Aims of our study is to evaluate the role of *CDKN2A* network in esophageal adenocarcinoma and to identify multiple targets for cancer therapy.

MATERIALS AND METHODS

Retrieval of CDKN2A PPI network from STRING database

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) a biological database, was used for retrieving the biological network of CDKN2A protein. STRING have predicted and known PPI interaction. It's also contains both the computational as well as experimental data that's are freely available source database and is updated regularly. The resource also focuses on function enrichment in user provided protein list. The latest version contains more than 9.6 million proteins from more than 2000 organisms (Ahmad et al 2020).

Identification of HUBS proteins in PPI network

The PPI network files was downloaded in PSI –XML format from String database and were upload to HUBBA analyzer for identification of hub proteins in CDKN2A network by using double screening method

Data pre-processing and Software's

In the current study, different bioinformatics databases, software's and tools were used to generate the network of *CDKN2A* associated with different types of Esophageal cancer. The network consist of many genes associated with human cancers. The gene list includes *PTEN, MCM10, MCM5, RRM2, CDKN2A, CDC7, HSP90AA1, CDC6, AURKA, MDM4, TUBA1A, TUBB4B, PML, NOP53, MYC, NPM1, CDK6, MDM2, PSME3, TUBB, CUL2, PPP1CB, ANAPC15, PCNA, MCM6, TP63, CCND2, SMARCA4, BRCA1, UBC, E2F1, CDK5RAP3, PSMC3, TP53, CCND1* and *CDK4*. R language was used to construct the map of cancer related genes in Clusterprofiler.

Enrichment analysis by using Clusterprofiler

In cluster profiler, Hyper geometric test was performed to evaluate the association of selected genes with *CDKN2A* and presented its result in the form of cnetplot and Dot plot. Cluster profiler use hyper geometric distribution to calculate the p-value for different analysis including enrichment analysis (Ito, Ohga et al. 2007).

Mathematical form of hyper geometric distribution is as below.

$$p = 1 - \sum_{i=0}^{k-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

Association of CDKN2A mutations with Esophageal Adenocarcinoma

cbioportal, an online cancer genome platform was used to evaluate the mutation of *CDKN2A* associated with Esophageal adenocarcinoma. cbioportal provides dataset in the form of oncoprint and mutation graphs. For survival analysis, Kaplan-Meier plots with a Logrank test was performed on 182 patient sample to generate overall survival rate, progression rate, and disease-specific and disease-free survival rate.

RESULTS

Enrichment Analysis using Hypergeometric test

PTEN, MCM10, MCM5, RRM2, CDKN2A,

CDC7, HSP90AA1, CDC6, AURKA, MDM4, TUBA1A, TUBB4B, PML, NOP53, MYC, NPM1, CDK6, MDM2, PSME3, TUBB, CUL2, PPP1CB, ANAPC15, PCNA, MCM6, TP63, CCND2, SMARCA4, BRCA1, UBC, E2F1, CDK5RAP3, PSMC3, TP53, CCND1 and *CDK4* genes play important roles in cell cycle including mitotic cell cycle phase transition, G1/S transition of mitotic cell cycle, cell cycle G1/S phase transition and regulation of mitotic cell cycle phase transition (Figure 1).

Hypergeometric tests calculate p-value for selected genes on the bases of its functions. In dot plot, dot are coloured on the bases of p-value. The p-value of blue dot is 2.521681e-15 while the p-value for red dots is 1.196235e-17. Dot plot show all selected genes play role in various biological process including lipid modification, lipid phosphorylation, peptidyl-tyrosine phosphorylation, protein kinase B signalling, protein auto phosphorylation, phosphatidylinositol metabolic process, phosphatidylinositol-3-phosphate biosynthesis process (Figure 2).

Network and oncoprints of CDKN2A in Esophageal adenocarcinoma

CDKN2A Protein- protein interaction network was retrieved from STRING database using gene name as a query. The network consist of 62 nodes, 596 edges, 0.734 averaging cluster coefficient while protein-protein interaction enrichment p-value is < 1.0e-16. Network show *MDM2, MYC, HRAS, CDKN1A, CCND1, CDK4, CDK6, RB1, E2F1* and *TP53* are the interactors of *CDKN2A* play important role in different biological process associated with CDKN2A signaling network (Figure 3). Interestingly, a close association between *CDKN2A* and *TP53* were observed in esophageal adenocarcinoma via network study.

Cancer Genomic Alteration summary

cbioportal was used to generate genomic alterations of *CDKN2A* and its interactors in the form of oncoprint summary. 171(94%) samples out 182 had alterations in *CDKN2A, MDM2, MYC, HRAS, CDKN1A, CCND1, CDK4, CDK6, RB1, E2F1* and *TP53*. 87% cases had missense and truncating mutations in *TP53* while 47% cases had deep deletion, missense mutations, and truncating mutations in *CDKN2A* (Figure 4). Co-occurrence of *CDKN2A* and *TP53* were observed in Esophageal adenocarcinoma during mutual exclusivity analysis in the form of statistics (Log Odds Ratio= 1.521, p-value= 0.024, q-value=0.270) (jan et al.2018; bashir, jan et al. 2021).

Mutations of CDKN2A and TP53 in Esophageal adenocarcinoma

Mutation studies reveal 180 *TP53* mutations in Esophageal adenocarcinoma patient samples, out of which 105 are missenses, 65 are truncating while 10 are in frame mutations located at different domain of *TP53 (*Figure 5). 15 mutations of *CDKN2A* including 7 missense, 7 truncating, 1 in frame were observed in 182 samples of Esophageal patients, 4 of them were X153-splice/ D153N (Figure 6). Somatic mutation frequency of *TP53* and *CDKN2A* mutation in Esophageal adenocarcinoma are 86.8% and 8.2%.

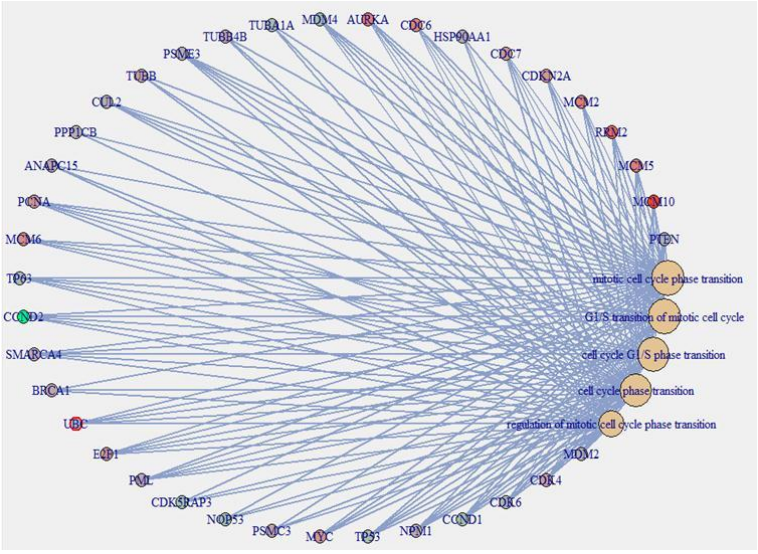

Figure 1: Cnet plot of 37 Genes associated with cancer via cluster profiler

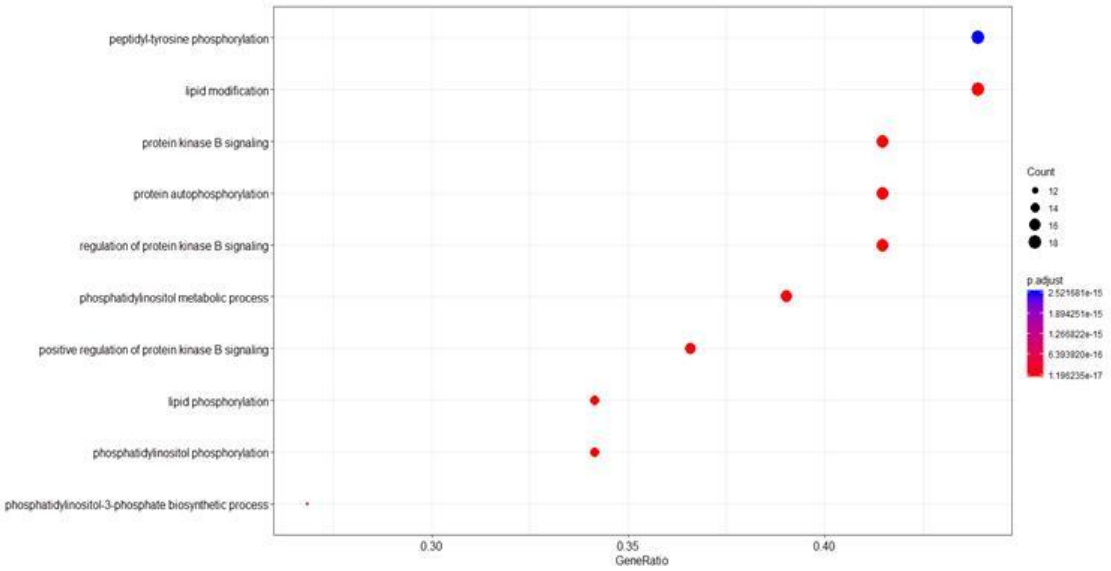

Figure 2: Dot plot along with p-value of 37 cancer associated genes via cluster profiler

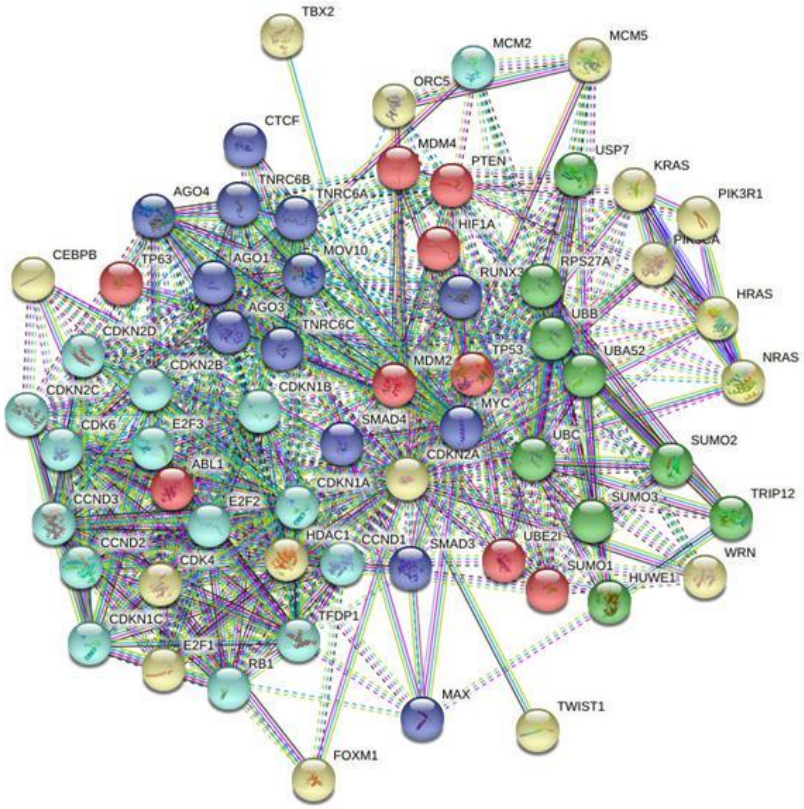

Figure 3: CDKN2A signalling network obtained from STRING Database

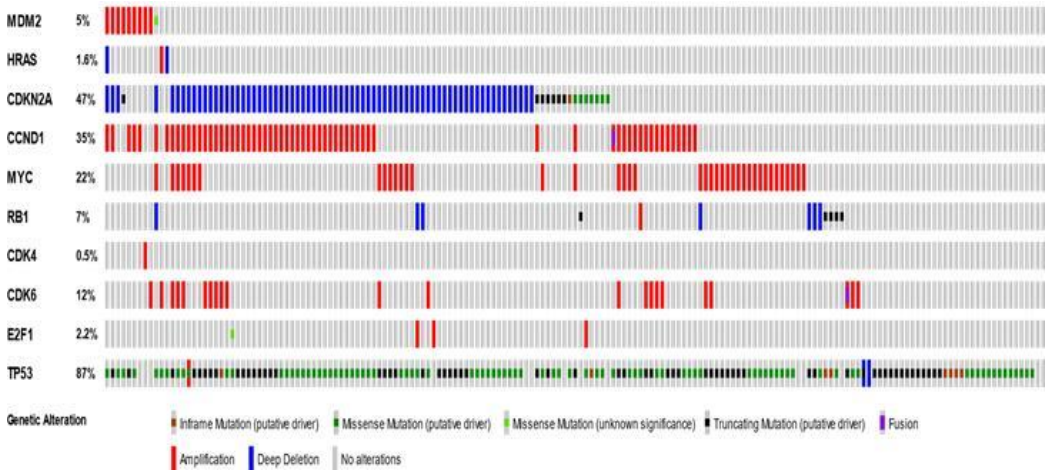

Figure 4: Oncoprint analysis of CDKN2A obtained from cbioportal

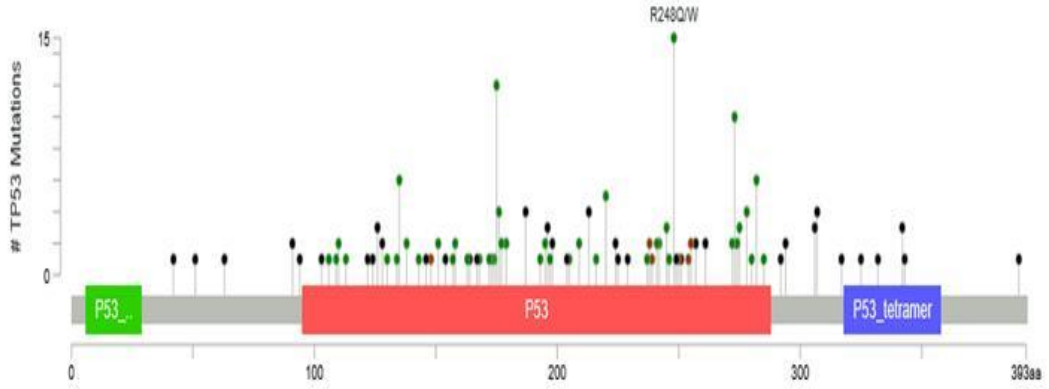

Figure 5: Mutation graph of TP53 in Esophageal Adenocarcinoma

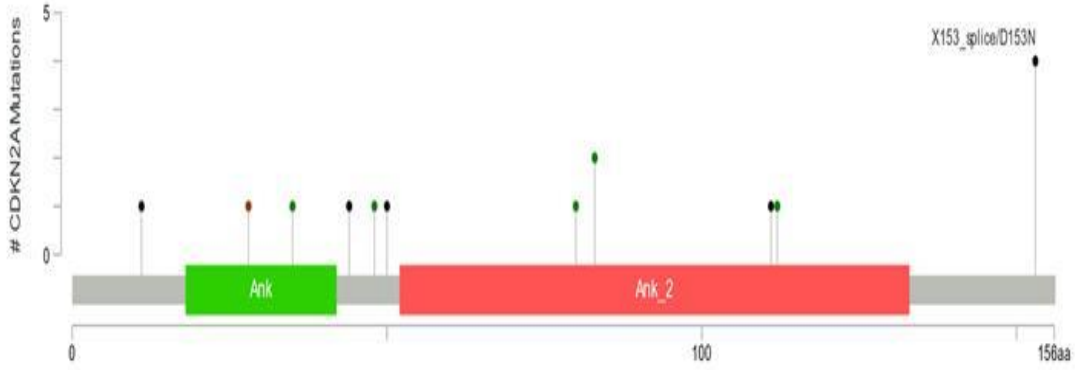

Figure 6: Mutation graph of CDKN2A in Esophageal Adenocarcinoma

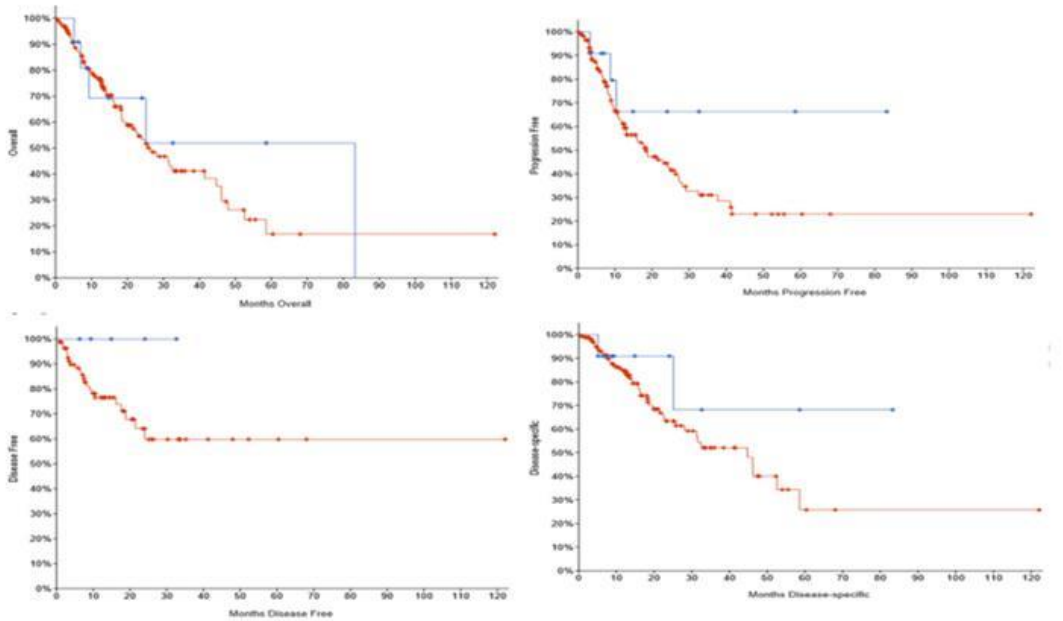

Figure 7: Survival analysis a) Overall survival b) Progression Free survival c) Diseases free Survival d) Diseases specific survival

Kaplian Meier plot with Logrank test for survival analysis

Logrank test was performed to generate plots of overall survival, progression-free survival, disease -free survival and disease specific survival. The p-value of overall, progression free, disease free and disease-specific survival is 0.463, 0.143, 0.212, 0.129, respectively. In the overall survival analysis, mutations in *CDKN2A* and *TP53* were present in 41.52% (71/171) of cases while in disease free, 26.8% (22/82) cases affected from mutations of *CDKN2A* and *TP53.* For progression free Kaplian Meier plot, 47.95% (82/171) cases affected from mutations of *CDKN2A* and *TP53.* In disease specific plot, mutations of *CDKN2A* and *TP53* were found in 28.4% (48/169) of cases (Figure 7).

DISCUSSION

Bioinformatics use in-Silico approaches to identified the location or specific gene, prediction of transcript of a particular genes and protein structure and location inside the cell (Bashir, Ahmad et al. 2021; Jan, Ahmad et al. 2021; Ahmad et al. 2021)

In the current study, we used comprehensive computational and statistic approaches to identify the role of *CDKN2A* signalling network in Esophageal adenocarcinoma. In *CDKN2A* network, 10 hub genes were identified, each of them performs various functions including lipid modification, lipid phosphorylation, peptidyl-tyrosine phosphorylation, protein kinase B signalling, protein auto phosphorylation, phosphatidylinositol metabolic process, phosphatidylinositol-3-phosphate biosynthesis process and show different mutations rate. This work reveal that *CDKN2A* mutate 47% cases related to Esophageal adenocarcinoma. Liu et al*.* reported that an initial incident in the ESCC carcinogenesis is associated with *CDKN2A mutations* (Liu et al. 2017). Promoter hypermethylation, inactivation and mutations of *CDKN2A* is highly testified in ESCC patients (Abbaszadegan, Raziee et al. 2005; Network 2017).

Zheng et al. reported that *TP53* is highly mutated gene in Esophageal cancer and he reported five mutations p.R175H, p.C176F, p.C176Y, H179R, p.S241F were found in L2-L3 zinc binding domain of *TP53* (Zheng et al. 2016). While in the present work, *TP53* show 87% mutations in 182 cases. *TP53* is tumor suppressor genes encode a protein called p53 which play important role in cell cycle regulation, DNA repairing, maintaining of genomic stability and apoptosis (Kastan et al. 1991; Levine and Oren 2009). Mutual exclusivity analysis reveals a rough association between *CDKN2A* and *TP53.* Our research reported, 22% mutations of *MYC,* 7% mutation of *RB1*, 12% mutation of *CDK6,* 2.2% mutation of *E2F1,* 5% mutations of *MDM2,* 1.6%

mutations of *HRAS,* 35% mutation of *CCND1* along with *CDKN2A* and TP53 were highly observed in 182 cases. *CCND1* is the third most common mutated gene in Esophageal adenocarcinoma.

CONCLUSION

In conclusion, our study provides strong indication that mutation in *CDKN2A* is associated with Esophageal adenocarcinoma. Proteins associated with *CDKN2A* also play important role in EC. We identify a close association between *TP53* and *CDKN2A* during network analysis. Mutation of *CDK6, TP53, MYC* and *CCND1* highly associated with *CDKN2A* signalling network and Esophageal adenocarcinoma. In future, experimental validation of this research could be employed for the identification of new diagnostic markers for esophageal cancer.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

H Sadia, Z Jan and S Umair designed the research, performed virtual screening and revised the manuscript. Khan MS, Khan MS, and Asad S, ullah K, performed the bioinformatics analysis, analyzed the virtual screening experiment data and drafted the manuscript. M Kamran, Irfan U, MK Kmaran checked the structures and made the Excel forms. All authors have read and approved the final manuscript.

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