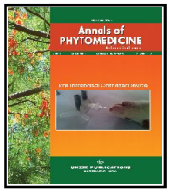
Annals of Phytomedicine, Special Issue 1, AU Pharmacon (TRIPS-2022): S10-S17, 2022

DOI: http://dx.doi.org/10.54085/ap.trips.2022.11.1.2

Annals of Phytomedicine: An International Journal
http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

The role of coumarin, hespertin and liquritigenin against oral cancer novel drug target FAP protein

Manal A. A. Moustafa* and John J. Georrge*,,◆**

**Department of Bioinformatics, Christ College, Rajkot (Affiliated to Saurashtra University, Rajkot), Gujarat-360005, India*
***Department of Bioinformatics, University of North Bengal, Raja Rammohunpur, Siliguri, District-Darjeeling, West Bengal-734013, India*

Article Info

Article history
Received 1 August 2022
Revised 5 September 2022
Accepted 6 September 2022
Published Online 30 October 2022

Keywords
Mutation pressure
Natural selection
RSCU
Down-regulated genes
Oral cancer
CUB analysis
Docking

Abstract

The study of codon usage bias (CUB) can be seen as a platform for identifying the gene composition, the effect of evolutionary forces and other traits influencing the codon usage pattern. It is natural for some codons to be chosen over others in gene coding sequences; however, some disorders may result from the quiet modification of some synonymous codons. One of the fatal diseases that has recently expanded over the world is oral cancer. According to certain research, oral cancer is linked to unhealthy practices in these areas, like smoking and chewing tobacco. Here, we investigated the CUB for 677 down-regulated oral cancer-related genes. The bases for the CDs were composed in the following order: C > G > A > T and the codons in the third-place are: C3 > G3 > T3 > A3. Genes were also GC-rich and the order of the three locations' total GC content was: GC3 > GC1 > GC2. For 677 genes, the effective number of codons and their values were computed and the average value was >35, indicating low CUB. The more commonly used codons were predominantly GC ending, according to RSCU values. Ten codons were under represented, while there were 2 over represented codons. Based on GC12 versus GC3 concentrations, the neutrality plot demonstrated the superiority of natural selection over directed mutation pressure in creating CUB. The study of the genes leads to a new target of oral cancer that FAP protein. Using docking tools, we could detect a good interaction between three natural products; coumarin, hespertin and liquiritgenin.

1. Introduction

Numerous diseases have emerged in this decade, with cancer making the most impact. According to estimates from the International Agency for Research on Cancer, updated by Globocan (2020), there were 19.3 million new cancer cases worldwide, with a death rate of close to 10 million. With almost 657,000 new instances of cancer in the oral cavity or pharynx reported each year and about 330,000 fatalities, oral cancer has emerged as one of the most common neoplasms in the world. Additionally, oral cancer has the highest fatality rate compared to other cancer types. In developing nations like Bangladesh, India, Pakistan and Sri Lanka, oral cancer is common and accounts for over one-fourth of all new cancer cases (Hung *et al*., 2020). The main causes of this elevated risk have been poor dental hygiene and alcohol and tobacco use. Geographical location, alterations in lifestyle, evolution and genetic factors such as cell cycle, DNA repair and cell death has increased the number of cases worldwide.

Squamous cell carcinomas account for over 90% of all oral cancer types' invasiveness; malignant cells can move to the deep regions of the oral cavity (Sciubba, 2001). According to their incidence, the eight different forms of oral cancer are classified as follows: buccal mucosa (32%), tongue (22%), lower lip (11%), palate (11%), the vestibule (8%), alveolus (5%), mouth floor (5%) and gingiva (3%). Men were shown to have a higher prevalence of major mouth cancer cases than women (D'Souza and Addepalli, 2018). Certain distinct symptoms seem to have emerged in probable leukoplakia in oral cancer patients. A white patch that forms in the mouth's lining and cannot be removed is called leukoplakia. Even so, it may continue to exist painlessly for years. Leukoplakia patients are more likely to have this illness (Lodi *et al*., 2016). Additionally, systematic primary, secondary and tertiary prevention of leukoplakia can be crucial in reducing mortality and morbidity due to inadequate diagnosis of oral squamous cell carcinoma. A histological classification system is a useful tool for early prognosis and planning treatment (Grajewski and Groneberg, 2009).

We set out to investigate the genetic makeup of oral cancer, one of the most prevalent diseases in the world and to describe the results of a codon usage bias (CUB) analysis of 677 down-regulated oral cancer-related genes. Apart from Met and Trp, other amino acids (18) typically transcribed by two or more codons are preferentially encoded by a few chosen codons, resulting in a bias, a phenomenon known as CUB. Different genes, genomes, animals and species exhibit the CUB phenomenon differently (Uddin *et al*., 2019; Mazumder*et al*., 2014). Studying CUB reveals to us the molecular genesis of genes. CUB can affect transcription, mRNA stability and all protein synthesis-related processes (Chamary *et al*., 2006). Knowing CUB could help demonstrate differentiating and evolution amongst species (Tang *et al*., 2021).

Some biological factors are related to CUB (tRNA abundance, GC content, gene expression and gene length) (Hu *et al*., 2014). Furthermore, there have been several hypothesised processes to explain why uncommon codons are abundant in the 5'-region of

Corresponding author: Dr. John J. Georrge
Associate Professor, Department of Bioinformatics, University of North Bengal, Raja Rammohunpur, Siliguri, District-Darjeeling, West Bengal-734013, India
E-mail: johnjgeorrge@gmail.com
Tel.: +91-9898609230

genes, suggesting that CUB may play a significant role in gene transfer and protein folding. The translational ramp (or bottleneck) is a region at the start of genes that maintains ribosome spacing uniformly to prevent collisions. That area might also experience sluggish translation (Hockenberry*et al*., 2014).

Codon use and tRNA abundance are two essential factors in controlling protein synthesis. Recently, scientists discovered that the amounts of tRNA are dynamically regulated in various biological circumstances. This might cause modifications to the transcript's codon composition-dependent translation efficiency (Benisty *et al*., 2020). To fully understand some characteristics, such as the co-adaptation of codon-anticodon and gene expression to meet the demanding secondary structural requirements, tRNA quantification is also necessary. The Cancer Genome Atlas (TCGA) study's impact on cancer translational machinery and tRNA gene expression is too important to ignore (Hernandez-Alias *et al*., 2020). As shown by CUB, it is evident that the influence of mutational pressure was predominant in human oncogenes (Barbhuiya *et al*., 2019).

A total of 677 down-regulated genes were found in this study using various criteria specified in the material and methods. The codon use pattern of these genes was then analysed. The risk of oral cancer is rising steadily, primarily due to unhealthy habits, including cigarette use, poor oral hygiene, lifestyle changes, and a few environmental problems. Thus, mouth cancer is the only form of cancer connected to human decisions. Surgery is the main choice among doctors because oral cancer has a high mortality rate and no known cure yet. Therefore, the codon usage pattern of the oral cancer genes was investigated to learn more about them. We have published gene expression, base composition, more and less common codon usage and over represented and under represented codons in the genes down-regulated in oral cancer from this study (Moustafa *et al*., 2022). To comprehend the CUB, many evolutionary factors and their effects on these genes were also illustrated. In terms of genetic makeup, we think this study will help determine a novel and successful treatment plan for oral cancer. Using docking tools was one of the greatest tools for drug discovery, working with phytomedicine products. The identified oral cancer potential target got interaction with some anticancer phytochemicals.

2. Materials and Methods

2.1 Data retrieval of oral cancer

The National Center for Biotechnology Information's (NCBI) Gen Bank database was used to extract the coding sequences of 677 down-regulated genes linked to oral cancer in FASTA form (https://www.ncbi.nlm.nih.gov).

2.2 The effective number of codons

The parameter effective number of codons (Chamary *et al*., 2006), which measures codon usage bias, ranges from 20 (maximum bias) to 61 (no bias). This number aids in determining the level of CUB in a gene's coding sequence. The minimum value of ENC is 20, which means only a single codon is used for each amino acid (severe bias) (extreme bias). ENC's highest score of 61 indicates that all codons are utilised equally (no bias) (Wright, 1990; Fuglsang, 2006).

$$ENC = 2 + \frac{9}{F_2} + \frac{1}{F_3} + \frac{5}{F_4} + \frac{3}{F_6}$$

where F_a(a = 2, 3, 4 or 6) is the average of the values of the amino acids with a-fold degeneracy.

2.3 Genomic variance in synonymous codon usage

Another measure known as the Relative Synonymous Codon Usage (RSCU) value of codons can be used to depict the difference in synonymous codon usage among genes. The ratio of the observed codon frequency to the anticipated codon frequency is determined using RSCU (Hassan *et al*., 2009; Sharp and Li, 1987).

RSCU value was determined for each codon by following the formula (Nayak, 2009): where, indicates the frequency of the codon for amino acid and is the number of codons for the amino acid (codon family).

2.4 Base content

Four nucleotide bases (A, T, C, and G) were calculated. Because the nucleotides at the third position of the codon are crucial to CUB, A3%, T3%, C3%, and G3% were identified. However, to identify the variations in codon positions, the relationship between the GC contents in the three positions of codons was investigated (Chakraborty *et al*., 2020).

2.5 Neutrality plot

A neutrality plot could show the relationship between G + C content at various codon positions (GC3 and GC12). The neutrality plot is a popular bioinformatic method for determining the equilibrium of mutation pressure and natural selection acting on genes. If the slope is equal to 1 and the connection between GC3 and GC12 is highly significant, it implies that mutation pressure has a greater impact on genes (He *et al*., 2016; Sueoka, 1999).

2.6 Molecular docking

By the full analysis of the effect of genes on oral cancer, we identified a new target, FAP protein, which is responsible for oral cancer and causes great pain in patients (Bhattacharya *et al*., 2020). The docking was performed between FAP protein and natural product phytomedicines ligands from the COCONUT database.

2.6.1 Ligand retrieval and preparation

Potential phytomedicine molecules were retrieved from the COCONUT database to perform docking. The COCONUT database is the most recent and unique natural product (NP) https://COCONUT.naturalproducts.net/. The COCONUT database contains natural products (NPs) from 53 resources, such as zinc NP, UNPD, CHEMBL NPs, Afro cancer, *etc*. The recent release of the COCONUT database comprises 4,06,076 unique (no stereochemistry) NPs, and 730,441 NPs with stereochemistry (Sorokina *et al*., 2021).

These ligands were prepared by using Lig Prep as a tool of Schrodinger software. Lig Prep 2.3 is used to take 2D structures and produce the corresponding low-energy 3D structures with correct chiralities. The OPLS-2005 force field was used for minimisation as an Epic ioniser at the standard pH of 5-9 which the maximum number of conformers per structure was 1000 with RMSD 1.0 A (Mahto *et al*., 2014).

2.6.2 Retrieval and preparation of protein 3D structures

FAP protein was retrieved from protein data bank (PDB) database (Berman *et al*., 2000). The PDB identification number of FAP protein is 1z68. Protein data bank is considered the most powerful global tool for 3D structure determination (https://www.rcsb.org/).

Protein 3D structure was prepared by using the protein preparation wizard from Schrodinger to optimise the fixed structure by locating and fixing structural defaults in a raw state, assigning bond orders, adding hydrogen, creating zero-order bonds to metals, creating disulfide bonds, filling in missing side chains, fill in missing loops, and deleting un-wanted chains, water and het groups (Sastry *et al*., 2013).

2.6.3 Grid generation

Active site prediction of all proteins was performed using the Prank Webtool (https://prankweb.cz/) (Jakubec *et al*., 2022).The OPLS-2005 force field was used to define the active site for receptor grid generation.

2.6.4 Natural products (COCONUT database)

Three potential phytomedicine molecules were retrieved from the COCONUT database to perform docking. The COCONUT database is the most recent and unique natural product https://COCONUT.naturalproducts.net/. The COCONUT database contains natural products (NPs) from 53 resources such as zinc NP, UNPD, CHEMBL NPs, Afro cancer, *etc*. The recent release of the COCONUT database comprises 4,06,076 unique (no stereochemistry) NPs, and 730,441 NPs with stereochemistry (Sorokina *et al*., 2021).

2.6.5 Docking studies

After identifying the protein's active site regions of the receptor, search for the different locations in which ligands could bind. Thus, Glide (Grid-based Ligand Docking with Energetics) was used to determine the docking process using hierarchical filters (Thomsen and Christensen, 2006).

3. Results

3.1 Nucleotide composition of down-regulated genes in oral cancer

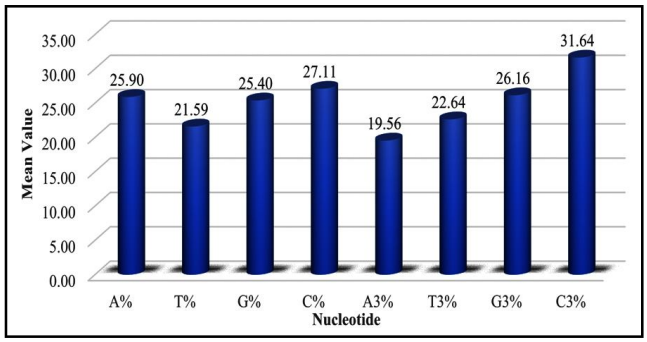


Figure 1: Nucleotide compositions of down-regulated genes in oral cancer.

Gene coding sequence characteristics affect CUB and impact total gene expression (Jenkins and Holmes, 2003). The nucleotide compositions were examined for 677 oral cancer genes that were down-regulated. Base frequency was found to be in the following order: C (27.12%), G (25.40%), A (25.90%) and T (21.59%). When GC and AT contents were compared in down-regulated genes, we discovered that they were 52.50% and 47.50%, respectively. This finding suggests that the genes are GC rich. C3 (31.64%) G3 (25.40%), T3 (22.64%), and A3 (19.56%) were the bases at the third codon position (Figure 1). Additionally, the three synonymous codon positions' individual GC contents were calculated, and it was found that position Three (37%) had the greatest GC content, followed by positions one (36%) and two (27%) (Figure 2).

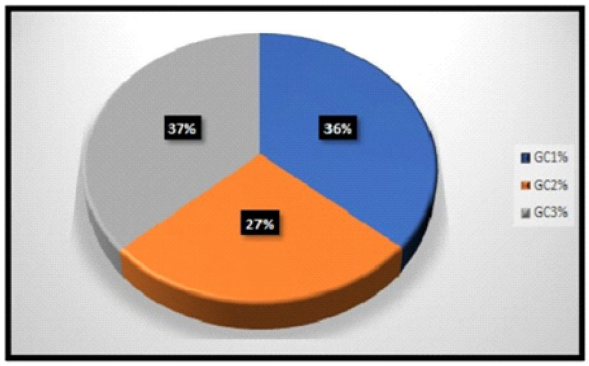


Figure 2: GC content analysis of down-regulated genes in oral cancer.

3.2 Detection of CUB based on ENC

For many down-regulated genes linked to oral cancer, the ENC value was computed to detect the level of CUB. Since the ENC number, in this case, ranges from 27 to 60, it is likely that there are significant genetic variances in the way codons are used. Nevertheless, given that the mean ENC value was 49.08 > 35, we may conclude that the CUB was generally low.

We, therefore, created an ENC-GC3 plot with the ENC value on the Y-axis and the GC3 contents on the X-axis. The solid line depicts the anticipated curve, with a few dots closer to the curve signalling that mutational pressure may have an impact and a few others spread randomly, demonstrating that natural selection may also impact the CUB of genes (Figure 3).

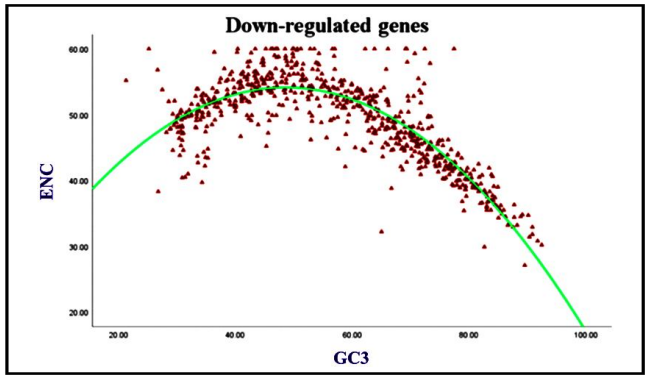


Figure 3: Relationship between GC3 and ENC (ENC plot).

3.3 Frequent usage of codons in down-regulated genes

All 677 down-regulated genes' RSCU values for synonymous codons were calculated (Figure 4). Except for two amino acids, Met (ATG) and Trp (TGG), represented by a single codon, all 18 amino acids are encoded by 59 synonymous codons. The codons were divided into four groups based on RSCU values: I over represented codons (>1.6), (ii) under represented codons (0.6), (iii) more frequently used codons (>1) and (iv) less frequently used codons (Quax *et al*., 2015). The over represented codons were CTG and GTG, whereas those that were under represented were TCG, TTA, CTA, CCG, CAA, CGT, ATA, ACG, and GTA. 21 codons were utilised less frequently, and 26 were more frequently.

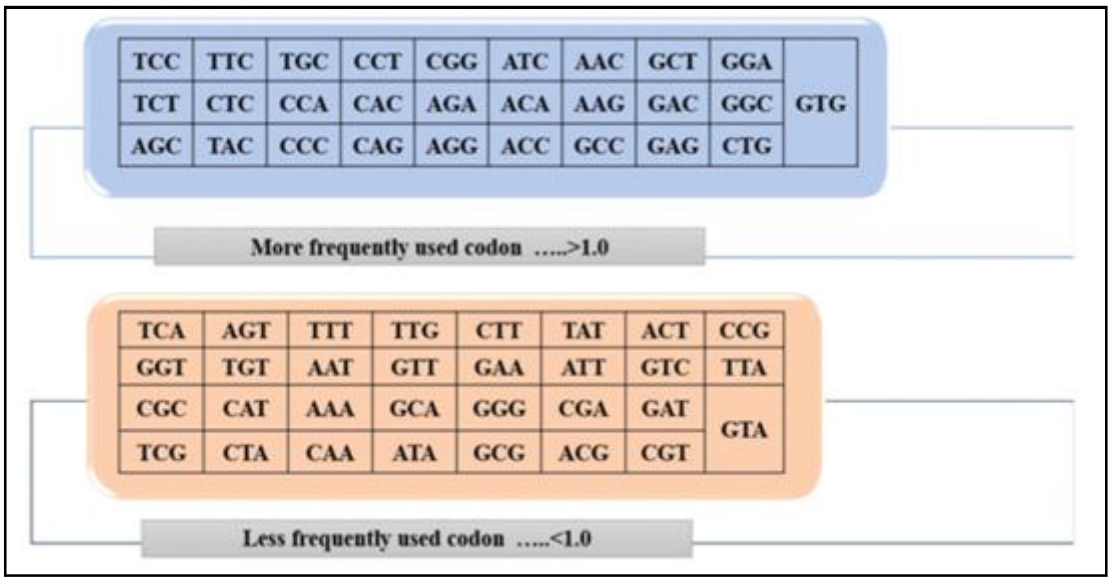


Figure 4: Codon usage frequencies of down-regulated genes in oral cancer.

3.4 Neutrality plot

The precise impact of mutational pressure and the natural section on the CUB of down-regulated oral cancer-related genes was quantified using the neutrality plot. We detected a significant positive association between the contents of GC12 and GC3, 0.605** at $p<0.01$, demonstrating that directed mutation impacted the CUB. Additionally, when we framed GC12 versus GC3 on a neutrality plot, as shown in Figure 5, we discovered that the regression coefficient value was 0.228, suggesting that mutational pressure played a 22.8% greater role in the evolution of genes than natural selection did. As a result, although mutational pressure also impacted the genes, natural selection was the primary evolutionary factor in determining CUB in down-regulated genes of oral cancer.

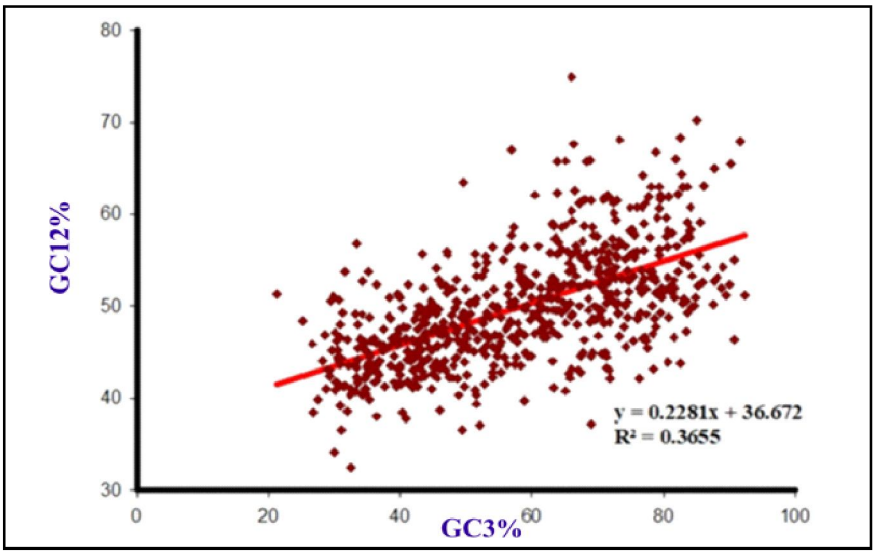


Figure 5: Neutrality plot of down-regulated genes in oral cancer.

3.5 Molecular docking

The docking score and the interacted amino acids between three natural products and oral cancer potential protein FAP are shown in Table 1 and Figure 6.

CNP0324043 is one of the coumarin derivatives that have an anticancer effect. Coumarin derivatives could be found in higher plants such as Rutaceae and essential oils of cinnamon bark, cassia leaf and lavender oil (Bhattarai *et al*., 2021). CNP0248322 is a natural flavonoid called hespertin found in lemons and oranges. Hespertin combined with doxorubicin shows cytotoxic and antimetastatic activity towards Her2 that expresses in breast cancer cells (Nurhayati *et al*., 2020). CNP0044609 is known as liquiritigenin, which could be found in Glycyrrhiza genus in the perennial plant, Legume family. It is an excellent anticancer product (Ramalingam *et al*., 2018).

All compounds have an anticancer effect which could inhibit the oncogenic pathway. The results showed these molecules could be a strong phytomedicine against oral cancer.

Table 1: The interaction between three phytomedicine and FAP protein (1z68) showing the interacting amino acids

No.	COCONUT ID	Docking score (kcal/mol)	Bond types of interaction with amino acids					
			H-bond	Halogen bond	Metal coordination	Pi-Pi stacking	Salt bridge	Pi-cation
1	CNP0324043	-10.509	ARG 123 GLU 203 GLH 204 TYR 745 TYR 541			TRP 621 TRP 623 TYR 660		
2	CNP0248322	-8.831	ASP 703 ARG 123 TYR 745				ASP 703	
3	CNP0044609	-8.438	ARG 123 TYR 656 GLN 539			HIP 734		

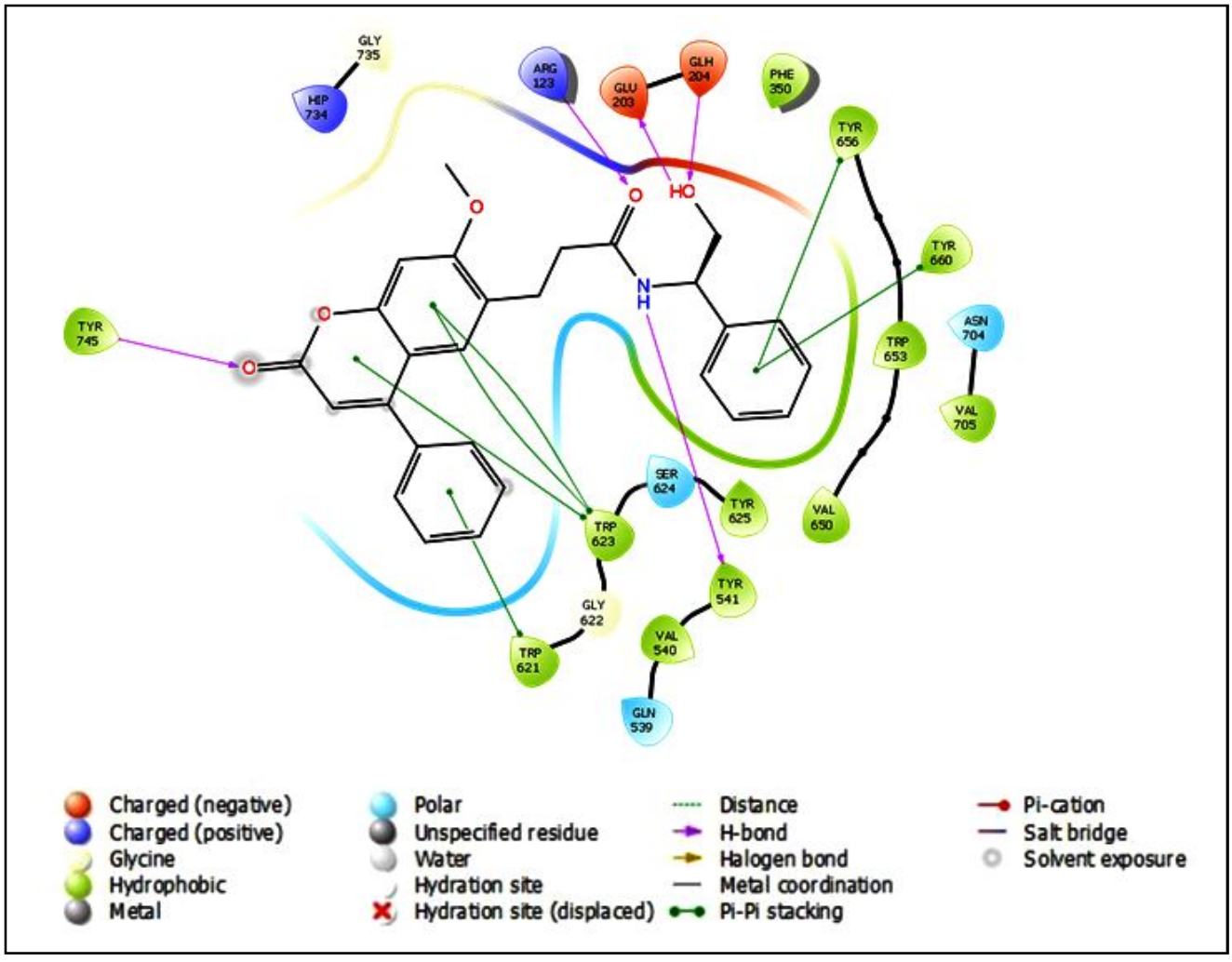


Figure 6 (a): The interaction between FAP protein and CNP0324043 showing the interacted amino acids.

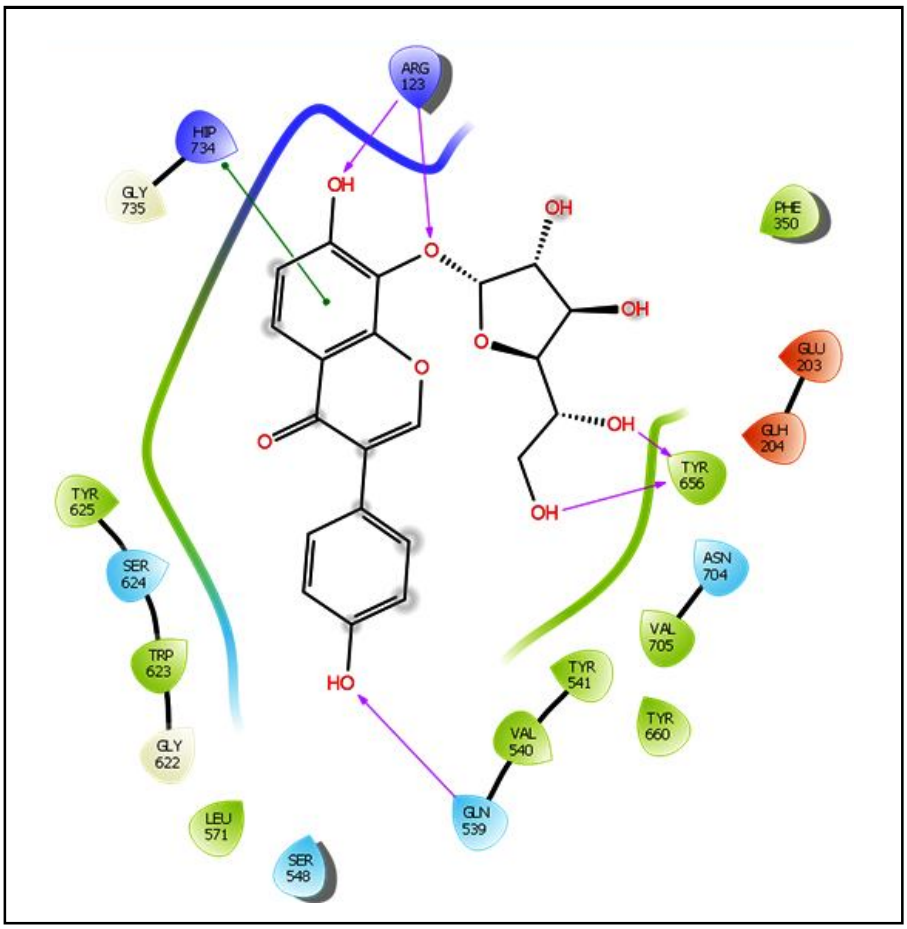


Figure 6 (b): The interaction between FAP protein and CNP0248322 showing the interacted amino acids.

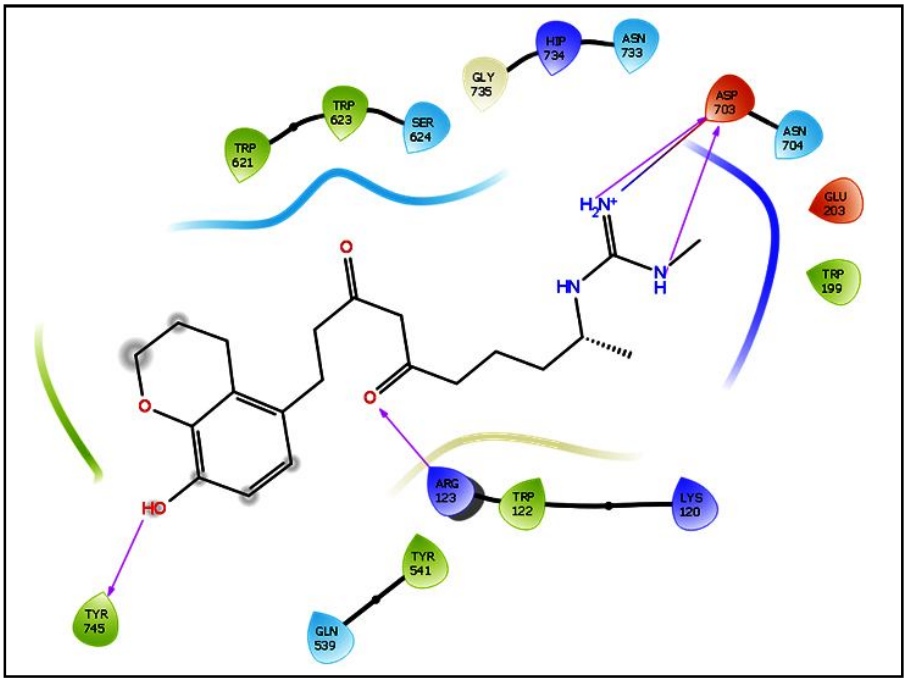


Figure 6 (c): The interaction between FAP protein and CNP0044609 showing the interacted amino acids.

4. Discussion

Because cancer is typically diagnosed late, it is frequently referred to as a quiet death. Multiple causes, including genetic modification, dietary changes, environmental conditions and others, might cause cancer cells to behave incorrectly. It is also a major concern that the inactivation of tumour suppressors and the activation of proto-oncogenes affect the development of tumours. One or both copies of suppressor genes may be deleted or turned inactive, resulting in mutation (Davenport *et al*., 2002). Some genes control the growth of mouth cancer and have the ability to transform a person's normal genetic makeup into a malignant one. Growth factor receptors (EGFRI c-erb1) and other **ras** family receptors may control the growth of oral cancer because it is a solid tumour that mostly forms on the epidermis (Jurel *et al*., 2014). This study analysed the down-regulated genes implicated in oral cancer metastasis to lymph nodes (Zhang *et al*., 2018). The coding sequences of 677 genes down-regulated in oral cancer were retrieved from National Center for Biotechnology Information (NCBI). In this study, CUB analysis was done on all the genes to understand the degree of codon bias, gene expression and compositional dissimilarities to identify the over represented and the under represented codons and to estimate the role of evolutionary forces in shaping their CUB.

The bases in the CDS were in the order C> G >A> T. The base contents in the third position of codons were C3> G3> T3> A3. This suggests that the genetic composition at the third position of codon maintained a relationship with the overall base composition of coding sequences. The GC contents at different codon positions were GC3 (57.80%), GC1 (36%) and GC2 (27%). Further, the correlations were highly significant between compositional constraints that suggested mutational bias might have played a role in CUB. A similar finding was revealed from the CUB study on the genes related to ovarian cancer (Uddin *et al*., 2019).

The ENC values of various down-regulated oral cancer genes showed a wide variation. Notably, the mean ENC value was higher than 35, indicating that the overall CUB of the genes was low and that synonymous codons generating amino acids in the coding sequences may be used with more flexibility. The ENC value was likewise higher than 35 when genes from the Coronaviridae family were studied. The outcome demonstrated that codon bias was negligible and that natural selection had little effect on the genes (Nyayanit *et al*., 2020). We found two over represented codons, ten under represented codons, 26 more frequently used codons and 21 less frequently used codons from the results of RSCU values for 59 codons in the current study (Supriyo Chakraborty *et al*., 2020).

Docking study is one of the effective tools for computer aided drug discovery as it saves time and money for discovering novel treatment leads. The results showed the interaction between a new oral cancer target (FAP protein) and three promising anticancer phytomedicine products (coumarin, hesperetin, and liquiritigenin). Coumarin derivatives are one of the potential anticancer agents due to the irrole in inhibiting tumour progression (Goud *et al*., 2020). Hesperetin and liquiritigenin are apoptosis inducers that could inhibit PI3K/AKt signalling pathway with low side effects. Research is ongoing on the effect of hesperetin on breast cancer (Liang *et al*., 2021; Nurhayati *et al*., 2020).

5. Conclusion

Oral cancer is one of the most lethal types of cancer, particularly in nations where poor habits such as chewing tobacco and drinking alcohol are prevalent. India has one of the highest incidences of oral cancer in the world. There is little information available for oral cancer. Thus, there is an investigation of 677 down-regulated genes connected to oral cancer and the many characteristics in this research report. Current studies identified three phytomedicines: coumarin, hespertin, and liquritigenin, as potential oral cancer inhibitors. This research will help researchers learn about oral cancer genes, enhance treatments and save more lives.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Citation **Manal A. A. Moustafa and John J. Georrge (2022). The role of coumarin, hespertin, and liquritigenin against oral cancer novel drug target FAP protein. Ann. Phytomed., Special Issue 1, AU Pharmacon (TRIPS-2022): S10-S17. http://dx.doi.org/10.54085/ap.trips.2022.11.1.2.**