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Original Article

Investigation of HPV DNA, in-silico validation and role of E6 protein in colorectal carcinogenesis

Investigação do DNA do HPV, validação *in silico* e papel da proteína E6 na carcinogênese colorretal

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Abstract

The human papillomavirus is a prevalent sexually transmitted disease. Studies have shown a connection between HPV and malignancies of the cervix, penis, vulva, vagina, anus, and oropharynx. Colon cancer ranks 10th among all cancers in Pakistan, with 2.7% incidence and 2.4% mortality, while rectal cancer ranks 16th with 1.8% incidence and 1.5% mortality, according to Globocan 2020 data. The native Pakistani population has been progressively affected by colorectal cancer in just four years. The frequency of the disease in males increased threefold, from 2.3 to 6.8%, and in females, from 2.5 to 6.7%. This study was based on 135 paraffin-embedded biopsy specimens collected from several hospitals in Hazara division, Pakistan. Predominantly, the samples were Adenocarcinoma of the colon, rectal squamous carcinoma, and adenoma of the colon. Ten normal biopsy specimens were taken as controls. After processing and treatment with liquid nitrogen, specimens were tested using the GeneXpert PCR to detect HPV DNA and high-risk genotypes HPV16, HPV18, and HPV45. No HPV DNA was found in any of the control samples. 85 (62%) of the 135 specimens had HPV DNA identified, while seven specimens were detected with low-risk genotypes HPV6, HPV11, and HPV55. In the In-silico study, the role of E6 in colorectal cancer development was investigated through pathway analysis. E6 protein structures were retrieved and visualized in MOE 2015.10. The structure of P16INK4A was also retrieved from Pdb, and docking was performed between E6 and P16INK4A to generate a docking complex.

Keywords: Colorectal, Human Papilloma Virus, Adenocarcinoma, adenoma, GeneXpert, Hazara Division, Pakistan.

Resumo

O papilomavírus humano (HPV) é uma doença sexualmente transmissível prevalente. Estudos mostraram uma conexão entre o HPV e malignidades de colo do útero, pênis, vulva, vagina, ânus e orofaringe. O câncer de cólon ocupa o 10º lugar entre todos os cânceres no Paquistão, com 2,7% de incidência e 2,4% de mortalidade, enquanto o câncer retal ocupa o 16º lugar com 1,8% de incidência e 1,5% de mortalidade, de acordo com dados do Globocan 2020. A população nativa do Paquistão foi progressivamente afetada pelo câncer colorretal em apenas quatro anos. A frequência da doença em homens aumentou três vezes, de 2,3 para 6,8%, e em mulheres, de 2,5 para 6,7%. Este estudo foi baseado em 135 espécimes de biópsia embebidos em parafina coletados de vários hospitais, na divisão de Hazara, Paquistão. Predominantemente, as amostras eram adenocarcinoma do cólon, carcinoma escamoso retal e adenoma do cólon. Dez espécimes de biópsia normais foram coletados como controles. Após o processamento e tratamento com nitrogênio líquido, os espécimes foram testados usando o GeneXpert PCR para detectar DNA do HPV e genótipos de alto risco HPV16, HPV18 e HPV45. Nenhum DNA do HPV foi encontrado em nenhuma das amostras de controle. Dos 135 espécimes, 85 (62%) tiveram DNA do HPV identificado, enquanto sete espécimes foram detectados com genótipos de baixo risco HPV6, HPV11 e HPV55. No estudo *in silico*, o papel do E6 no desenvolvimento do câncer colorretal foi investigado por meio da análise de vias. As estruturas da proteína E6 foram recuperadas e visualizadas no MOE 2015.10. A estrutura do P16INK4A também foi recuperada do Pdb, e o encaixe foi realizado entre E6 e P16INK4A para gerar um complexo de encaixe.

Palavras-chave: Colorretal, vírus do papiloma humano, adenocarcinoma, adenoma, GeneXpert, Divisão Hazara, Paquistão.

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1. Introduction

The large intestine, also known as the big bowel or colon, is a component of the digestive system and comprises the colon and rectum. The ascending colon refers to the first section. It all begins in the cecum, a structure that resembles a pouch and absorbs food from the small intestine (Siegel et al., 2023). It rises the right side of the stomach (belly), with the transverse colon referring to the second section. It moves from the right side to the left side of the body. The descending colon gets its name from the fact that it moves downward on the left. The fourth part is known as the sigmoid colon because of its "S" shape. The sigmoid colon, which joins the anus, is connected to the rectum. The proximal colon is composed of the ascending and transverse colon portions. Descending and sigmoid colons comprised the distal colon (Siegel et al., 2023). The inner lining of the colon or the rectum is where colorectal cancer begins as a tumour (Marley and Nan, 2016).

The danger of cancer spreading to other anatomical areas increases if this abnormal growth, known as a polyp, subsequently develops into the disease. It may first become a tumour on the colon or rectum wall before developing into blood or lymph vessels (Valastyan and Weinberg, 2011). More than 95% of colorectal malignancies are adenocarcinomas, the most common type of cancer. These start in the mucus-producing glands of the colon and rectum (Marley and Nan, 2016).

With over 200 distinct genotypes and a high prevalence, the human papillomavirus (HPV) is primarily spread from person to person through sexual activity (Gabutti et al., 2021). The most common type of neoplastic cancer in Asia is colorectal cancer, which affects both men and women equally. It accounts for 9.7% of all malignancies, apart from non-melanoma skin cancer. More than 50% of cases are found in industrialized nations worldwide, which can be attributed to a fast-paced way of life and a significant change in dietary practices (Esmeeta et al., 2022). Both sexes are prone to HPV-related pathological illnesses, which can vary from benign conditions like warts to cancers of the cervix, vagina, vulva, anus, penis, and oropharynx (Gabutti et al., 2021).

More than 200 different kinds of HPV exist, some of which are cancer-causing. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are some of the more prevalent varieties (Lynch et al., 2015). HPVs encode two oncoproteins, E6 and E7, which play a vital role in the development of HPV-induced carcinogenesis. They accomplish this cooperatively by focusing on distinct cellular pathways that regulate cell cycle, apoptosis, and cell polarity regulatory networks (Tomaić, 2016). The more often developing malignant tumors in both genital sites are undoubtedly associated with the HPV16 genotype, which is the most prevalent globally (Lynch et al., 2015). Epidermal or mucosal epithelial cells infected with the human papillomavirus (HPV) cause neoplasms, both benign and malignant. For instance, HPV16, 18, 31, and 45 are frequently found in anogenital cancers, particularly cervical and anal cancer; as a result, they are classified as high-risk or carcinogenic. These HPVs infect a cancer cell by integrating their viral genome into its DNA. Other HPV strains, such as HPV6 and HPV11, seldom cause anogenital malignancies and benign anogenital warts, and are low-risk or non-oncogenic (Zur Hausen, 2002).

There have been many studies done on colorectal cancer, which is currently thought to be a common disease. In 2011, Lorenzo and colleagues conducted a meta-analysis of multiple papers regarding the relationship between HPV and colorectal cancer, which had been published in the preceding 20 years. The researchers found a connection between cancer and HPV (Lorenzon et al., 2011). The study was conducted in the Chinese population to investigate colorectal cancer and found that HPV DNA was prevalent in tumour tissues compared to the non-tumour colorectal tissue and peripheral blood samples (Liu et al., 2011). Another study conducted in 2007 by Damin *et al.* receded the effect of HPV infection in colorectal cancer and prognostic variables (Damin et al., 2007). Out of 72 malignant colorectal samples, HPV DNA was found to be positive in 60 (83.3%) samples. No HPV DNA was found in any of the noncancerous.

The International Agency for Research on Cancer issued the GLOBOCAN 2018 evaluation of cancer incidence and mortality, which shows that there were 881,000 deaths linked to colorectal cancer (CRC) and more than 1.8 million new instances of the disease in 2018 with an incidence rate of 10.2% and a mortality rate of 9.2%, CRC is ranked third overall among all cancer types (Bray et al., 2018; Pilleron et al., 2019).

The primary objectives of current research are to investigate the presence and genotypic distribution of human papillomavirus (HPV) colorectal cancer cases through the GeneXper System. In addition, in silico validation of PCR results and the role of E6 in viral oncogenesis has also been confirmed.

2. Methodology

Study Limitations: 135 biopsy specimens were used in this study because colorectal biopsy specimens are difficult to obtain without any approved consent. Secondly, few hospitals deal with the processing of these kinds of samples. The majority of patients move to other cities in the country. The study population is limited to the Hazara Division only, with a population of 6188736.

2.1. Sample collection

The research was conducted at Abbott Laboratory, a BSL-3 private laboratory in Abbottabad, Khyber-Pakhtunkhwa province (Pakistan). A total of 135 paraffin-embedded tissue biopsy specimens with histological confirmation were obtained; these specimens comprised 65 colons, 25 ascending colons, 20 descending colons, 15 rectal polyps, 10 rectal biopsies, and 10 normal colorectal tissue samples (Figure 1). Samples were collected from Abbott Laboratory, various hospitals in the Hazara Division and the histopathology department of Ayub Medical College (Abbottabad).

Figure 1. Paraffin-embedded tissue biopsy. After gross examination of the biopsy, the best part of the tissue is selected and put in a tissue rack, and melted paraffin is poured on it.

2.2. Histopathological examination

2.2.1. Cutting

Each tissue was labelled before being sliced into sections using a microtome (Leica RM 2135 Germany) initially at 30 microns, then at 20, 10, and 5 microns for rough cutting. Subsequently, thin sections were cut ranging in size from 2 to 3 microns (Figure 1Sa). The best parts were submerged in distilled water and picked using a glass slide (Figure 1Sb). Following treatment with 70% alcohol, tissues were immersed for one to two minutes at 37° C in a tissue path (Figure 1Sc). The slides were warmed at 60 to 65°C on a slide warmer for 30 minutes. For ten minutes, the slides were submerged in xylene; after that, 80% 90% alcohol was added, and ultimately, 100% alcohol for 30 minutes to fix the slide.

2.2.2. Haematoxylin and eosin staining

After being cleaned with tap water and dried, the slides were stained with hematoxylin for 15 minutes. The dark blue-purple dye hematoxylin stains nucleic acids through a convoluted mechanism that is still not entirely understood. Eosin is a pink stain that indiscriminately stains proteins. The nuclei of normal tissue are stained blue, whereas the extracellular matrix and cytoplasm are stained pink to varying degrees. Subsequently, slides were rinsed with tap water again and dipped into 1% acid alcohol two to three times. Following the second washing, slides were dipped two to three times in 5% Eosin, then soaked in 80% alcohol for 30 minutes, then 90%, and lastly, 100% alcohol for 30 minutes. The slides were treated with alcohol and then placed in xylene for 30 minutes (Figure 2).

2.2.3. Mounting

Coverslips were placed on each slide after DPX (Dibutyl phthalate Polystyrene Xylene) oil had been applied and gently pressed to release any trapped air. The slide was then examined under a 100x microscope (Figure 3a). After being exposed to liquid nitrogen, each specimen was ground into a fine powder using a mortar and pestle (Figure 3b). After that, each crushed specimen was put into the supplied preserved Cyst solution (Figure 3c) and vertex, then 1 ml of solution was poured into an HPV cartridge and placed within the GeneXpert device after the assay completion findings were acquired.

Figure 2. The prepared slides were treated with Haematoxylin & Eosin Stains.

2.2.4. Sample Processing for GeneXpert

The wax was removed from each paraffin-embedded specimen using xylol (MERCK, Germany), and then the specimens were washed and labelled. Each specimen underwent a liquid nitrogen treatment before being ground into a fine powder using a mortar and pestle Fig.3S. The meshed specimen was mixed with the solution of preserved cyst and vertex, and a further 1 ml sample was loaded in an HPV cartridge and placed in the GeneXpert system. Results were taken upon completion of the assay.

2.2.5. GeneXpert System

Using real-time polymerase chain reaction, the GeneXpert Dx system automates target sequence detection in complex and simple samples, nucleic acid amplification, and sample preparation (PCR). The system delivers

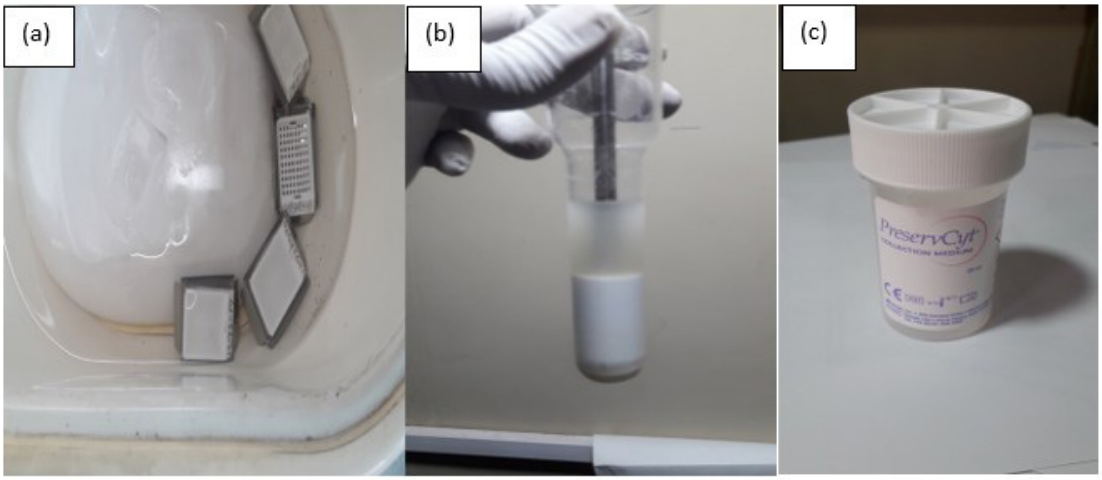

Figure 3. a) Dewaxing of paraffin-embedded tissue sample, b) Sample preparation for GeneXpert is done by treating it with liquid nitrogen. c) The crushed sample was added to the Preserved cyst solution.

tabular and graphic data for summarised and complete test findings. It was introduced for in vitro diagnostic applications where patient samples (specimens) must be processed via handoff. GeneXpert cartridges intended for single-use assays were used to prepare and process the samples. A cartridge containing the sample and reagents were inserted into the instrument module.

2.2.6. In silico study

In the *In silico* study, the role of E6 in colorectal cancer development was investigated through pathway analysis. E6 protein structures was retrieved from the protein database (PDB) (RCSB Protein Data Bank, n.d.) and visualized in MOE 2015.10. The structure of P16INK4A was also retrieved from Pdb, and docking was performed between E6 and P16INK4A to generate a docking complex. Docking will be performed using HDock (http://hdock.phys.hust.edu.cn/). The E6 protein pathway was retrieved and analysed in Reactome.

3. Results

3.1. Histopathological findings

The histopathological study verifies that, out of 65 colon biopsy specimens, Adenocarcinoma was detected in 35 specimens, carcinoma in 25, and squamous cell carcinoma in 10 specimens (Figure 4 a1,2 & 4b1,2). Similarly, adenocarcinomas were found in 15, carcinoma in 7 ascending colon samples (Figure 4 c1 &2), and 3 with adenomas. On the other hand, descending colon adenocarcinoma was detected in 13 specimens (Figure 4 d1 &2), metaplasia was detected in 4 (Figure 4e), and carcinoma in 3 samples. In addition, adenoma was found in 8, tubular villous adenoma with severe dysplasia was detected in 4 specimens, and carcinoma was found in 3 samples of the rectal polyp. In rectal specimens, 4 were detected with Adenocarcinoma, 3 with squamous cell carcinoma (Figure 4 f1 &2), and 3 with metaplasia, as shown in Table 1.

On the other hand, when 135 specimens were tested by GeneXpert, 85 (62%) were detected with HPV DNA, 37 (43%) with HPV-16, 25(29%) with HPV-18, and 16(19%) with HPV-45 genotype. Just 7 specimens had low-risk genotypes found, while 43 samples tested negative for HPV DNA; no detections were found in the normal control specimens, as shown in Table 2. Our results showed that HPV contributes significantly to cancer of the colon and rectum; therefore, it is advisable to consider it in colorectal cancer, like cervical cancer.

3.2. Insilco validation

3.2.1. Target structure retrieval

Structures of E6 (6siv) protein were retrieved from pdb (RCSB Protein Data Bank, n.d.) and visualized in MOE. A database that includes the three-dimensional structural information of giant biological molecules like proteins and nucleic acids is called the Protein Data Bank (pdb). However, MOE (Molecular Operating Environment) is a software platform for drug discovery that combines methodological development, modelling, and simulations into a single package.

3.2.2. Refined structure of E6

The E6 protein is a viral oncoprotein encoded by the human papillomavirus (HPV). It is an essential regulator of cellular functions, and its role in cancer is to interfere with the normal processes that control cell growth and proliferation (Figure 5). The structure of the E6 protein was refined using MOE. Chain B was kept while all other chains, water molecules, and ligands were removed.

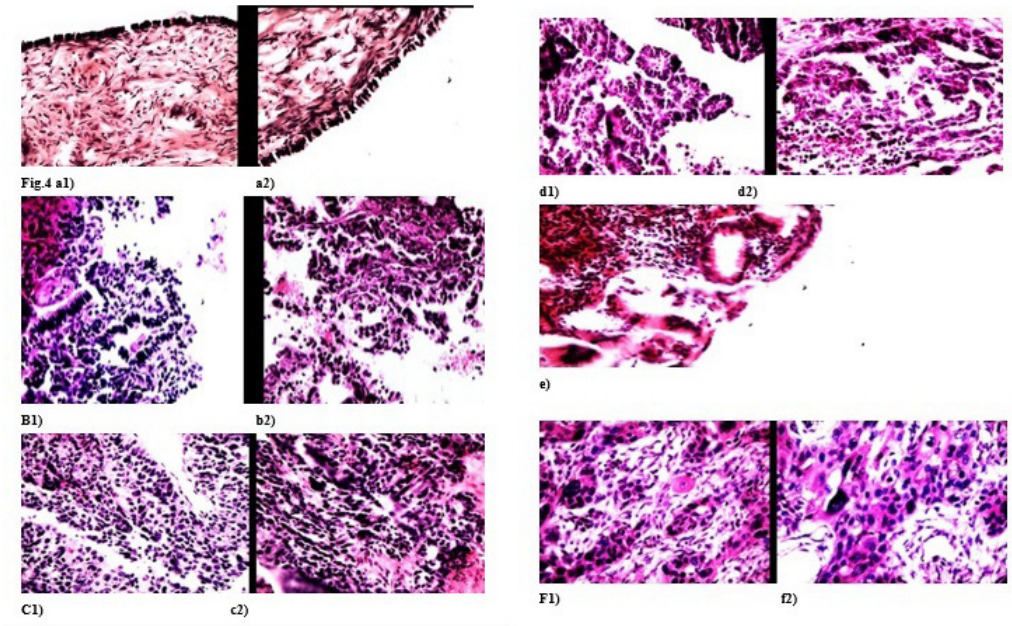

Figure 4. a1,2 & b1 &2): The figure in the slide depicts Adenocarcinoma of the colon, **c1 & c2)** shows the slide with Adenocarcinoma of the descending colon, **d1 & d2)**. The figure shows carcinoma of the ascending colon, **e)** slides show the metaplasia of the intestine, **f1 & 2).** The figure shows the slides with squamous cell carcinoma.

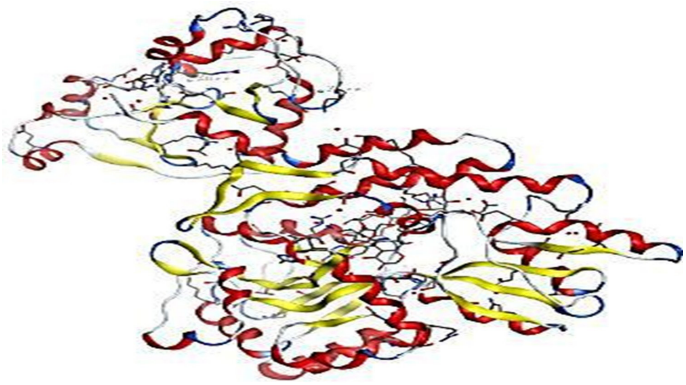

Figure 5. Structure of HPV16 E6 oncoprotein in complex with mutant IRF LxxLL motifs.

Table 1. Histopathological Investigation of Colorectal Biopsies.

No. Samples	Type	Conditions with Numbers and percentage	
65	Colon biopsy	Adenocarcinoma	35 (53.8%)
		Carcinoma	25 (38.4%)
		Squamous Cell Carcinoma	10 (15.3%)
25	Ascending Colon	Adenocarcinoma	15 (60%)
		Carcinoma	7 (28%)
		Adenoma	3 (12%)
20	Descending Colon	Adenocarcinoma	13 (65%)
		Metaplasia	4 (20%)
		Carcinoma	3 (15%)
15	Rectal Polyps	Adenoma	8 (53%)
		Tubulovillous adenoma	4 (26%)
		Carcinoma	3 (20%)
10	Rectal Biopsy	Adenocarcinoma	4 (40%)
		Metaplasia	3(30%)
		Squamous Cell Carcinoma	3 (30%)

Table 2. The results show the HPV DNA detection done by GeneXpert.

HPV	No	%	Genotype	No	%
Detected	85	62	HPV 16	37	43
			HPV 18	25	29
			HPV 45	16	19
Detected	7	5.1	Low-Risk HPV		
			HPV6 03		
			HPV11 02		
			HPV55 02		
Not Detected	43	31.8	HPV DNA not detected		
Control	10				
Total	135				

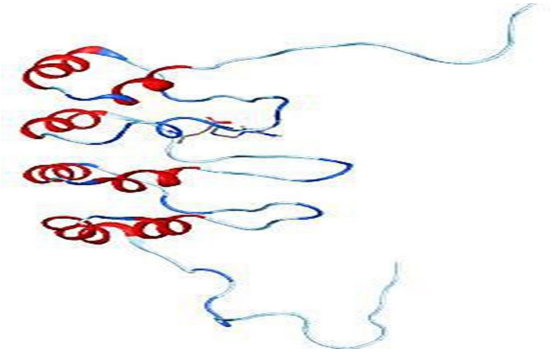

Figure 6. Structure of P16INK4A.

Figure 7. Dock complex of P16INK4A.

3.2.3. Aliases Of CDKN2A

A list of aliases of the CDKN2A gene was taken from BioGRID (The BioGRID, n.d.). BioGRID is a freely accessible database of physical and genetic interactions.

ARF, CDK41, CDKN2, CMM2, INK4, INK4A, MLM, MTS1, P14, P14ARF, P16, P16INK4, P16INK4A, P19, P19ARF, TP16 are the aliases of CDKN2A gene.

3.2.4. Structure of P16INK4A

p16INK4A is a tumour suppressor protein and part of the INK4 family of proteins. It comprises two domains: an N-terminal domain and a C-terminal domain. The structure of p16INK4A was also retrieved from pdb (Figure 6).

3.2.4. Dock complex of E6 and P16INK4A

The docking process involves predicting a ligand's preferred orientation toward a target when coupled to create a stable complex. Protein-protein docking was performed using HDock (http://hdock.phys.hust.edu.cn/). HDock supports protein-protein and protein-DNA/RNA docking and accepts both sequence and structure inputs for proteins. This analysis can be used to gain insight into the molecular mechanisms of P16INK4A and E6 and their interactions with other proteins. H Dock generated several models; the best one was selected based on the docking score. The dock score of our model was -246.54 (Figure 7).

3.2.6. Protein-protein interactions

The interaction between the tumour suppressor protein, p16INK4A, and the human papillomavirus oncoprotein, E6, is essential in cancer biology. E6 binds to and interacts with p16INK4A, leading to its degradation in the cell and thus allowing for uncontrolled cell proliferation. This interaction is essential for the development and progression of many types of cancers, including cervical cancer and head and neck cancer. Thus, understanding the mechanism of this interaction can help in the development of targeted therapies for these cancers. Protein-protein interactions of the docked complex were checked using Pdbsum, which shows that the number of hydrogen bonds was 4 and the number of non-bonded contacts was 156 as shown in (Figure 8).

3.2.7. Networks of aliases

Biological Networks are used to represent systems as complex sets of binary interactions or relations between distinct biological entities. The networks of Aliases of CDKN2A were analysed using String (http://www.thebiogrid.org), Gephi 0.9.7 (a Java-based open-source network analysis and visualization tool), cityscape 3.9.1 (open-source software platform allows users to visualize intricate networks and combine them with various kinds of attribute data), and BioGRID (http://www.thebiogrid.org).

3.2.8. From STRING

The aliases obtained from BioGRID were written in the string database, and it returned the network. The PPI enrichment p-value of 0.0439 indicates that there is a statistically significant enrichment of edges in the network compared to a random network with the same number of nodes (10) and edges (14). The average node degree of 2.8 and the average local clustering coefficient of 0.773 suggest that the network is moderately dense, and the nodes are clustered. The expected number of edges is 8, which is lower than the actual number of edges in the network, indicating that the network is more densely connected than expected (Figure 9).

3.2.9. From Gephi and BioGRID

The Gephi network shows the interactions between the two proteins E6 and P16INK4A. E6 is a protein associated with human papillomavirus (HPV) that is known to play a role in promoting cancer. P16INK4A is a tumour suppressor

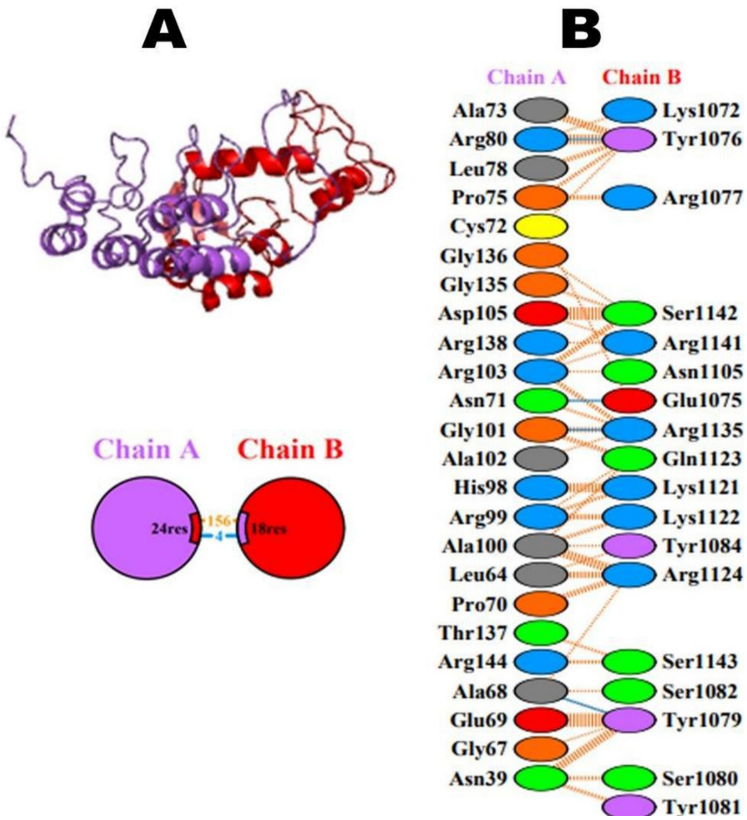

Figure 8. The quantity of possible hydrogen bonds between any two residues is indicated by the number of H-bond lines that are present between them. The width of the striped line is proportional to the number of atomic contacts in the case of non-bonded interactions, which can occur in large quantities.

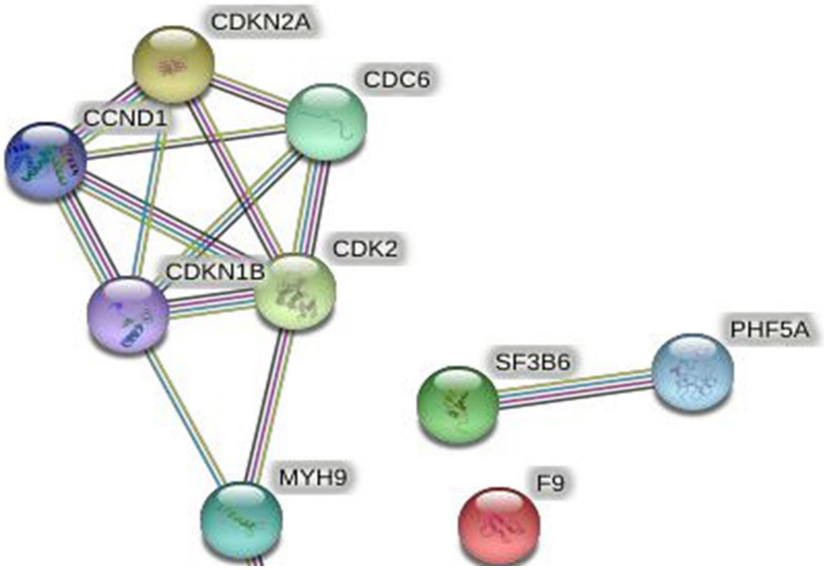

Figure 9. STRING network showing interacting partners of E6 and P16INK4A.

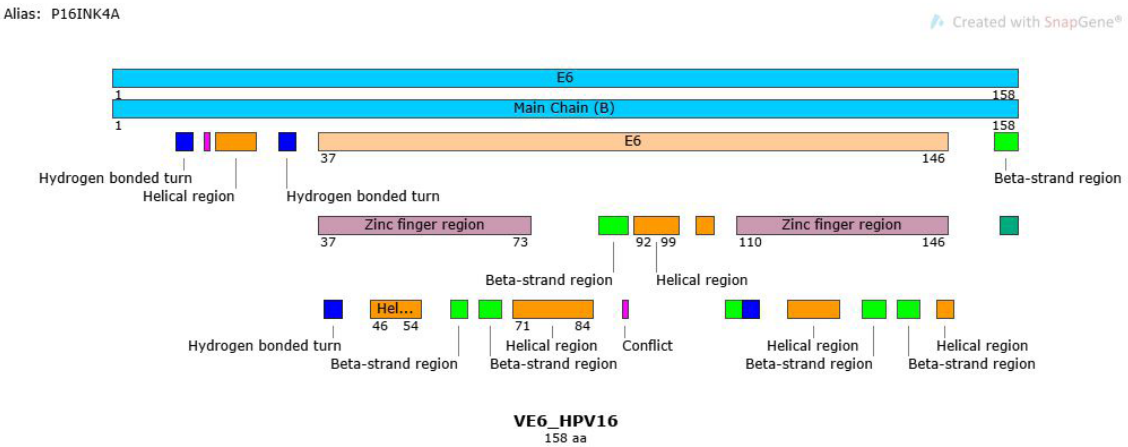

Figure 10. Graphical representation of HPV16 Sequence Map from SnapGene.

gene that is involved in regulating the cell cycle and can act to counteract the effects of E6. The arrows indicate that E6 activates P16INK4A, while the circles denote that P16INK4A inhibits the activity of E6. The network was selected by searching the aliases. It showed only one network that was involved in colorectal cancer. The network showed 73 edges and 188 nodes with a directed graph (Figure 6S).

The ability of the CDKN2A gene to bind to CDK4 and CDK6 is compromised by missense and nonsense mutations, which produce amino acid changes in p16INK4A or p16INK4A truncations, respectively (Figure 10). These mutations also obstruct p16INK4A's ability to induce cellular senescence in response to oxidative stress. Loss-of-function mutations in p16INK4A can also contribute to cancer by interfering with p16INK4A-mediated inhibition of NFKB signalling.

4. Discussion

The current study's findings strongly suggested that even if HPV is not the only element contributing to the expansion of colorectal cancer, it may still be considered one of the diseases causes as reflected in our results, HPV DNA was detected in paraffin-embedded biopsy specimens which were cancer positive in the histological study 62% positive HPV DNA was detected by using GeneXpert PCR system. Because extraction and amplification are entirely automated in cartridges and the GeneXpert system also detects the genotype involved in a single run, we were able to employ it in our study without worrying about human mistakes. Even though PCR equipment used by other studies differed from ours, we have demonstrated that HPV-16 is the most common genotype found in our cases (Ibragimova et al., 2018).

According to the previous study (Rezaei et al., 2020), papillomavirus, which is frequently detected in human tumours and may play a role in cancer development, is responsible for 15% of all current cases of cancer. The same findings were presented in a study conducted in Iran in 2015 (Mahmoudvand et al., 2015), and the same results are reflected in our research, as the most prevalent type is HPV-16. These results stimulate further investigation into the virus' presence in larger populations, its involvement in viral oncoprotein expression during oncogenesis mutations in HPV-positive tumours, and colon infection mechanisms (hematologic/lymphatic spreading or perineal diffusion).

Another study proposed that preventive actions, including vaccination and the use of antiviral drugs appropriate for the affected communities' health plan, should be taken because HPV is a risk factor for colorectal cancer (Li et al., 2015). According to new research, oncogenic viruses are thought to be the root cause of 15% of all malignancies. Papillomavirus is frequently found in human tumours and can contribute to cancer development (Aran et al., 2016). These reports again satisfy our results, although multiple factors are involved like population, race, ethnicity, living style, and habits; despite these differences, we have almost similar results.

According to the research study (Qiu et al., 2020), cervical cancer has been largely verified clinically as being caused by HPV, and cervical cancer prevention and therapy have significantly advanced thanks to the anti-HPV vaccine. Our research has shown that the tissues of colon cancer contain HPV DNA. The same results were shown in a study conducted (Qiu et al., 2020) that the tissues of colon cancer contain HPV viral antigens. It is crucial to determine the link between HPV infection and the development of CRC cancer. They showed in this investigation that tumour tissues had more HPV antigen than nearby non-neoplastic tissue. Our findings strongly suggested that HPV may contribute to CRC carcinogenesis via gene mutation. More genomic and proteomic research is required for a more thorough understanding of the signalling pathways connected to CRC carcinogenesis.

A Meta-analysis showed that it became evident there is a link between colorectal cancer and HPV. Expressive values were observed in the prevalence of HPV (51.8%), especially for types 16 and 18, due to late diagnosis, seeing that the confirmation of the disease is more frequent in stage III. Another factor that was observed is the lack of evidence in the differences between genders regarding

the prevalence of colorectal cancer due to HPV, seeing that both sexes presented similar values. During the research period of this study, colorectal cancer owing to HPV was diagnosed in 51.8% of cases. Of these, the majority were linked to HPV 16 and 18, with tumour prevalence in the cervical area and similarity between genders. Therefore, HPV infection, as nowadays it is one of the most common sexually transmitted diseases worldwide and as it is linked to colorectal cancer, has become an essential method in early diagnosis for the prevention of new cases. It also enables new studies to facilitate the prognostic and treatment of the disease (Pelizzer et al., 2016).

These days, HPV has been shown to have a role in colorectal carcinogenesis. The transportation of HPV to the colon is not always clear. The ascending contamination from the perianal area, sexual abuse, fomites inoculation, or colonoscopy-like procedures can be the sources of HPV contamination within the colon (Deschoolmeester et al., 2010) P16INK4A is a sensitive marker for HPV. In certain clinical contexts, enhanced p16ink4a expression significantly predicts treatment response and illness prognosis. High-level expression of p16ink4a in tumours is linked to aggressive subtypes of the disease. The frequency of p16INK4A immunohistochemically expression varies in wonderful research and levels from 17% to 80% (Dalla Libera et al., 2020). P16ink4a expression in colorectal carcinoma is 82%. P16 protein is upregulated stepwise in colorectal adenoma and colorectal carcinoma. Our investigations proved that the p16INK4A protein is over-expressed in colorectal cancer cases. However, HPV-associated carcinogenesis must be confirmed by detecting HPV DNA in host cells.

The current study has addressed two different aspects regarding high-risk human papillomavirus. There is insufficient data on the prevalence and genotyping of HPV in Pakistani society. The first part of the study was to detect HPV DNA and find out the prevalent HPV subtype in colorectal biopsies. Although this study was performed on a relatively smaller sample size, it still provides baseline information about HPV prevalence in the Hazara Division of Pakistan. High-risk gene variants, it was discovered that HPV16 was the most common subtype (Santos et al., 2022). Variant analysis is another tool that may be used in future research to assist in determining the precise evolutionary relationships.

The second part of this study was to study the role of E6 protein in cancer development. The study's findings suggest that HPV infection is closely related to the marker Protein p16INK4A encoded by E6 in colorectal cancer patients. Also, there is evidence that p16INK4A may be a possibility for the creation of biomarkers for the diagnosis and prognosis of colorectal cancer based on the differential expression between colorectal cancer tumour tissues and normal controls, as well as its association with overall survival (Al-Grawi and Al-Awsi, 2018). In the *In silico* study, we showed that interaction between the tumour suppressor protein, p16INK4A, and the human papillomavirus oncoprotein, E6, is important in cancer biology. E6 binds to and interacts with p16INK4A, leading to its degradation in the cell and thus allowing for uncontrolled cell proliferation. This interaction is essential for the development and progression of many types of cancers, including cervical cancer and head and neck cancer (Dalla Libera et al., 2020). Thus, understanding the mechanism of this interaction can help in the development of targeted therapies for these cancers. Protein-protein interactions of the docked complex were checked using PDBsum shows that the number of hydrogen bonds was 4 and the number of non-bonded contacts was 156. Moreover, we found that there are different Aliases of the CDKN2A gene, and E6 and E7 viral proteins that can interact and cause abnormal proliferation in tissues (Shi et al., 2022). More genomic and proteomic research is required for a more thorough understanding of the signalling pathways connected to CRC carcinogenesis.

Our findings support the hypothesis that HPV infection and CRC/adenoma share some disease links, which may indicate that further study of the relevant mechanism of HPV and the development of HPV-related vaccine may have great significance for the prevention of CRC. We conclude that cervical cancer caused by HPV has received widespread clinical confirmation and that cervical cancer prevention and treatment have significantly improved through the anti-HPV vaccine. In our research, we discovered that colon cancer tissues contain HPV viral antigens. It is important to study the link between HPV infection and the development of CRC cancer. This work showed that neighbouring non-neoplastic tissue did not contain the exact quantities of High-risk HPV antigen as tumour tissues did.

References

AL-GRAWI, E.D.C. and AL-AWSI, G.R.L., 2018. Expression of CDKN2A (P16/Ink4a) among colorectal cancer patients: a cohort study. *Journal of Pharmaceutical Sciences and Research*, vol. 10, pp. 1145-1147.

ARAN, V., VICTORINO, A.P., THULER, L.C. and FERREIRA, C.G., 2016. Colorectal cancer: epidemiology, disease mechanisms and interventions to reduce onset and mortality. *Clinical Colorectal Cancer*, vol. 15, no. 3, pp. 195-203. http://doi.org/10.1016/j.clcc.2016.02.008. PMid:26964802.

BRAY, F., FERLAY, J., SOERJOMATARAM, I., SIEGEL, R.L., TORRE, L.A. and JEMAL, A., 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a Cancer Journal for Clinicians*, vol. 68, no. 6, pp. 394-424. http://doi.org/10.3322/caac.21492. PMid:30207593.

DALLA LIBERA, L.S., DE SIQUEIRA, T., SANTOS, I.L., PORTO RAMOS, J.E., MILHOMEN, A.X., ALENCAR, R.D.C.G.D., RABELO SANTOS, S.H., DOS SANTOS CARNEIRO, M.A., FIGUEIREDO ALVES, R.R. and SADDI, V.A., 2020. Detection of Human papillomavirus and the role of p16INK4a in colorectal carcinomas. *PLoS One*, vol. 15, no. 6, pp. e0235065. http://doi.org/10.1371/journal.pone.0235065. PMid:32584870.

DAMIN, D.C., CAETANO, M.B., ROSITO, M.A., SCHWARTSMANN, G., DAMIN, A., FRAZZON, A., RUPPENTHAL, R.D. and ALEXANDRE, C.O.P., 2007. Evidence for an association of human papillomavirus infection and colorectal cancer. *European Journal of Surgical Oncology*, vol. 33, no. 5, pp. 569-574. http://doi.org/10.1016/j.ejso.2007.01.014. PMid:17321098.

DESCHOOLMEESTER, V., VAN MARCK, V., BAAY, M., WEYN, C., VERMEULEN, P., VAN MARCK, E., LARDON, F., FONTAINE, V. and VERMORKEN, J.B., 2010. Detection of HPV and the role of p16INK4Aoverexpression as a surrogate marker for the presence of functional HPV oncoprotein E7 in colorectal cancer. *BMC*

Cancer, vol. 10, pp. 117. http://doi.org/10.1186/1471-2407-10-117. PMid:20346145.

ESMEETA, A., ADHIKARY, S., DHARSHNAA, V., SWARNAMUGHI, P., MAQSUMMIYA, Z.U., BANERJEE, A., PATHAK, S. and DUTTAROY, A.K., 2022. Plant-derived bioactive compounds in colon cancer treatment: an updated review. *Biomedicine and Pharmacotherapy*, vol. 153, pp. 113384. http://doi.org/10.1016/j.biopha.2022.113384. PMid:35820317.

GABUTTI, G., D'ANCHERA, E., DE MOTOLI, F., SAVIO, M. and STEFANATI, A., 2021. Human Papilloma Virus Vaccination: focus on the Italian Situation. *Vaccines*, vol. 9, no. 12, pp. 1374. http://doi.org/10.3390/vaccines9121374. PMid:34960120.

IBRAGIMOVA, M.K., TSYGANOV, M.M. and LITVIAKOV, N.V., 2018. Human papillomavirus and colorectal cancer. *Medical Oncology (Northwood, London, England)*, vol. 35, no. 11, pp. 140. http://doi.org/10.1007/s12032-018-1201-9. PMid:30187207.

LI, Y.X., ZHANG, L., SIMAYI, D., ZHANG, N., TAO, L., YANG, L., ZHAO, J., CHEN, Y.Z., LI, F. and ZHANG, W.J., 2015. Human papillomavirus infection correlates with inflammatory Stat3 signaling activity and IL-17 level in patients with colorectal cancer. *PLoS One*, vol. 10, no. 2, pp. e0118391. http://doi.org/10.1371/journal.pone.0118391. PMid:25706309.

LIU, J., DUAN, Y., CHENG, X., CHEN, X., XIE, W., LONG, H., LIN, Z. and ZHU, B., 2011. IL-17 is associated with poor prognosis and promotes angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma. *Biochemical and Biophysical Research Communications*, vol. 407, no. 2, pp. 348-354. http://doi.org/10.1016/j.bbrc.2011.03.021. PMid:21396350.

LORENZON, L., FERRI, M., PILOZZI, E., TORRISI, M.R., ZIPARO, V. and FRENCH, D., 2011. Human papillomavirus and colorectal cancer: evidences and pitfalls of published literature. *International Journal of Colorectal Disease*, vol. 26, no. 2, pp. 135-142. http://doi.org/10.1007/s00384-010-1049-8. PMid:20809427.

LYNCH, H.T., SNYDER, C.L., SHAW, T.G., HEINEN, C.D. and HITCHINS, M.P., 2015. Milestones of Lynch syndrome: 1895–2015. *Nature Reviews. Cancer*, vol. 15, no. 3, pp. 181-194. http://doi.org/10.1038/nrc3878. PMid:25673086.

MAHMOUDVAND, S., SAFAEI, A., ERFANI, N. and SARVARI, J., 2015. Presence of human papillomavirus DNA in colorectal cancer tissues in Shiraz, Southwest Iran. *Asian Pacific Journal of Cancer Prevention*, vol. 16, no. 17, pp. 7883-7887. http://doi.org/10.7314/APJCP.2015.16.17.7883. PMid:26625815.

MARLEY, A.R. and NAN, H., 2016. Epidemiology of colorectal cancer. *International Journal of Molecular Epidemiology and Genetics*, vol. 7, no. 3, pp. 105-114. PMid:27766137.

PELIZZER, T., DIAS, C.P., POETA, J., TORRIANI, T. and RONCADA, C., 2016. Colorectal cancer prevalence linked to human papillomavirus: a systematic review with meta-analysis. *Revista Brasileira de Epidemiologia*, vol. 19, no. 4, pp. 791-802. http://doi.org/10.1590/1980-5497201600040009. PMid:28146168.

PILLERON, S., SARFATI, D., JANSSEN-HEIJNEN, M., VIGNAT, J., FERLAY, J., BRAY, F. and SOERJOMATARAM, I., 2019. Global cancer incidence in older adults, 2012 and 2035: a population-based study. *International Journal of Cancer*, vol. 144, no. 1, pp. 49-58. http://doi.org/10.1002/ijc.31664. PMid:29978474.

QIU, Q., LI, Y., FAN, Z., YAO, F., SHEN, W., SUN, J., YUAN, Y., CHEN, J., CAI, L., XIE, Y., LIU, K., CHEN, X. and JIAO, X., 2020. Gene expression analysis of human papillomavirus-associated colorectal carcinoma. *BioMed Research International*, vol. 2020, pp. 5201587. http://doi.org/10.1155/2020/5201587. PMid:32258125.

REZAEI, M., MOSTAFAEI, S., AGHAEI, A., HOSSEINI, N., DARABI, H., NOURI, M., ETEMADI, A., NEILL, A.O., NAHAND, J.S., MIRZAEI, H., DONNELLY, S.C., DOROUDIAN, M. and MOGHOOFEI, M., 2020. The association between HPV gene expression, inflammatory agents and cellular genes involved in EMT in lung cancer tissue. *BMC Cancer*, vol. 20, no. 1, pp. 916. http://doi.org/10.1186/s12885-020-07428-6. PMid:32972386.

SANTOS, N.C., TOCANTINS, P.D.B., LEÃO-CORDEIRO, J.A.B., ATAIDES, F.S., MARQUES, L.D.O.R. and SILVA, A.M.T.C., 2022. The human papillomavirus in colorectal cancer. *Yixue Yanjiu Zazhi*, vol. 42, no. 1, pp. 1-7. http://doi.org/10.4103/jmedsci.jmedsci_194_20.

SHI, W.-K., LI, Y.-H., BAI, X.-S. and LIN, G.-L., 2022. The cell cycle-associated protein CDKN2A may promotes colorectal cancer cell metastasis by inducing epithelial-mesenchymal transition. *Frontiers in Oncology*, vol. 12, pp. 834235. http://doi.org/10.3389/fonc.2022.834235. PMid:35311137.

SIEGEL, R.L., WAGLE, N.S., CERCEK, A., SMITH, R.A. and JEMAL, A., 2023. Colorectal cancer statistics, 2023. *CA: a Cancer Journal for Clinicians*, vol. 73, no. 3, pp. 233-254. http://doi.org/10.3322/caac.21772. PMid:36856579.

TOMAIĆ, V., 2016. Functional roles of E6 and E7 oncoproteins in HPV-induced malignancies at diverse anatomical sites. *Cancers (Basel)*, vol. 8, no. 10, pp. 95. http://doi.org/10.3390/cancers8100095. PMid:27775564.

VALASTYAN, S. and WEINBERG, R.A., 2011. Tumor metastasis: molecular insights and evolving paradigms. *Cell*, vol. 147, no. 2, pp. 275-292. http://doi.org/10.1016/j.cell.2011.09.024. PMid:22000009.

ZUR HAUSEN, H., 2002. Papillomaviruses and cancer: from basic studies to clinical application. *Nature Reviews. Cancer*, vol. 2, no. 5, pp. 342-350. http://doi.org/10.1038/nrc798. PMid:12044010.

Supplementary Material

Supplementary material accompanies this paper.

Figure: 1Sa. shows the cutting of tissue samples in a define range 1Sb) best tissue slice was submerged in distilled water 1Sc) immersed in 70% alcohol and left for 2-3 minutes at 37 °C.

Fig.3S. The sample preparation for the GeneXpert for grinding tissue using mortar and pestle

Figure 6S: Network representation E6 is a protein associated with human papillomavirus (HPV) that is known to play a role in promoting cancer.

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