Glycogene expression profiles from a HaCaT cell line stably transduced with HPV16 E5 oncogene

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Abstract. The altered expression of glycan antigens has been reported during cervix transformation, demonstrating increased mRNA levels of certain glycogenes. Human papillomavirus (HPV) is the aetiologic agent of cervical cancer. High risk HPV E5 is considered an oncogene and has been implicated in cell transformation. E6 and E7 HPV oncoproteins modify the expression of certain glycogenes. The role of the E5 HPV protein in glycogene expression changes has not yet been reported. The aim of the present study was to determine the effects of HPV16 E5 oncoprotein on glycogene expression. For these, a microarray assay was performed using the HaCaT cell line and altered glycogenes were identified. The mRNA levels of certain glycogenes were determined via reverse transcription-quantitative PCR (RT-qPCR). Using in silico analysis, the present study identified that glycosylation pathways were altered by E5. Microarray analysis revealed alterations in certain glycogenes, including the upregulation of ST6GAL1, ST3GAL3, CHST2, and MANBA, and the downregulation of UGT2B15, GALNT11, NDST2, and UGT1A10. Increased mRNA levels were confirmed via RT-qPCR for sialyltransferases genes. Additionally, in silico analysis was performed to identify glycosylation networks altered in the presence of the E5 oncoprotein. The analysis revealed that E5 could modify glycian sialylation, the N-glycosylation pathway, keratan sulfate and glycosaminoglycan synthesis. To the best of our knowledge, the current study was the first to determine the role of the HPV16 E5 oncoprotein in glycogene expression changes. The results indicated that increased sialyltransferase mRNA levels reported in pre-malignant and malignant cervical tissues could be the result of E5 oncoprotein expression. The results provide a possible role of HPV infection on glycosylation changes reported during cervix transformation.

Introduction

Human papillomavirus (HPV) is the aetiologic agent of cervical cancer (CC) (1). HPV16 is the most prevalent genotype and is responsible for greater than 50% of CC cases worldwide (2,3). The oncogenic potential of HPV is attributed to the following three viral proteins: E6 and E7, the best characterized viral proteins, promote cell transformation by several mechanisms; for example, they destabilize and induce the degradation of the tumour suppressor proteins p53 and pRB, respectively (4). Meanwhile, E5, a transmembrane protein present in the Golgi apparatus, endoplasmic reticulum and nuclear envelope (5), has several roles in cell transformation. For example, E5 can force cells through the cell cycle and promote the evasion of the immune response (6). Additionally, E5 can modify gene expression; HPV16 E5 induces the expression of prostaglandin E2 receptor by stimulating the binding of the cAMP-response element binding protein (CREB) to its promoter, which activates mitogenic activated protein kinase (MAPK) and increases C-FOS and C-JUN transcription (7,8). Moreover, E5 increases the expression of genes related to cell adhesion, cell motility, and mitogenic signaling, suggesting that the protein plays an important role in the events associated with cellular transformation (9).

Altered glycosylation is another characteristic of cancer cells (10), and it can be caused by the altered expression of different glycosyltransferases that can lead to changes in glycan structures. Increased mRNA levels of some sialyltransferases have been reported in different cancer types (11). Specifically, CC and premalignant lesions display increased mRNA levels of
the sialyltransferases $ST3GAL3$ and $ST6GAL1$ (12,13), which are related to increased expression of sialic acid (14,15) and of sialylated antigens such as sTn and sLe(x) (16-18). Studies in the HeLa cell line show that E6/E7 HPV18 oncogene knockdown modified glycogene expression, some of which participate in the synthesis of O-glycans such as fSfn (19). These results suggest that viral infection could modify glycogene expression and the glycosylation of the cervical epithelium.

The objective of this work was to identify glycogenes that displayed modified expression patterns in the presence of the HPV16 E5 oncprotein. The results showed that the HPV16 E5 oncprotein could increase the expression of the sialyltransferases $ST3GAL3$ and $ST6GAL1$, which have been reported to be altered in premalignant and malignant cervical tissues. The network interaction constructed with the altered glycogenes in the presence of E5 showed that not only the glycan sialylation but also some glycan structures, such keratan sulphate and glycosaminoglycans, could also be altered.

Materials and methods

Cell culture. The HaCaT cell line from human skin keratinocytes stably transfected with the HPV16 E5 oncogene (HaCaT-E5) (20) or the vector pMSG (HaCaT-pMSG) (kindly donated by Dr. A. Alonso from German Cancer Research Centre, University of Heidelberg, Germany) was cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) containing Earle's salts and L-glutamine (DMEM; Sigma-Aldrich; Merck KGaA) and supplemented with 10% foetal bovine serum (Biowest), 100 µg/ml streptomycin (Sigma-Aldrich; Merck KGaA). The CasKi cell line from a squamous CC (kindly donated by Dr A. Aguilar-Lemarroy from Centro de Investigación Biomédica de Occidente, IMSS) was cultured and maintained in RPMI1640 medium (Sigma-Aldrich; Merck KGaA) containing L-glutamine and supplemented with 10% foetal bovine serum, and 100 µg/ml streptomycin (Sigma-Aldrich; Merck KGaA). Cells were maintained at 37°C with an atmosphere of 5% CO$_2$. The culture medium was replaced every two days. Sub-confluent cells were harvested using a mixture of trypsin (0.025%) and edTa (Sigma-aldrich; Merck KGaA) and were washed with phosphate-buffered saline.

Microarray expression assay. The microarray expression assay was performed at the Cellular Physiology Institute of UNAM. The microarray contained 10,000 gene-specific oligonucleotide probes representing the best-annotated genes from human. For the probe preparation, total RNA from HaCaT/E5 and HaCaT/pGSM monocultures was obtained with the NucleoSpin II RNA kit (Macherey-Nagel).

Expression analysis of E5 and glycogenes. Total RNA from CasKi, HaCaT-E5 and HaCaT-pMSG monocultures was obtained with the NucleoSpin II RNA kit (Macherey-Nagel). The objectives of this work were to identify glycogenes that displayed modified expression patterns in the presence of the HPV16 E5 oncogene. The results showed that the HPV16 E5 oncprotein could increase the expression of the sialyltransferases $ST3GAL3$ and $ST6GAL1$, which have been reported to be altered in premalignant and malignant cervical tissues. The network interaction constructed with the altered glycogenes in the presence of E5 showed that not only the glycan sialylation but also some glycan structures, such keratan sulphate and glycosaminoglycans, could also be altered.

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Identification of glycogenes. For the analysis of the glycogenes in the microarray displaying altered expression, we considered 336 glycogenes reported to date using the GlycoGene database (http://riodb.ibase.aist.go.jp/rcmg/ggdb/), the Consortium for Functional Glycomics-CAZY database (http://www.cazy.org/CAZY/) and the published reports on glycogenes not included in the databases, including $DPY19L1$ (21) and MANBAL (22). We identified glycogenes displaying altered expression in the microarray as those with a z-score >2.

Protein-protein interaction network. The downregulated or upregulated set of glycogenes in the HaCaT-E5 cells were submitted separately, to the STRING database (http://string-db.org/). The following parameters were applied for the analysis: text mining, experiments, databases, co-expression,
neighbourhood, gene fusion and co-occurrence as interaction sources, no more than 5 interactor, minimum interaction score of 0.9 as confidence level, and a protein-protein enrichment P-value at least ≤0.05 and FDR at least <0.05.

Statistical analysis. Statistical analysis of the qPcr results was performed using the GraphPad program. The Student’s t-test was performed. A P-value <0.05 was considered statistically significant.

Results

E5 oncogene expression by RT-qPCR. RT-qPCR was performed to quantify E5 mRNA expression levels from HaCaT-E5 cells, as a positive control it was used mRNA of CasKi cells. HaCaT-pMSG was used as a negative control. Data are presented as the mean ± SEM (n=3).

E5 increased the expression of the sialyltransferases ST3GAL3 and ST6GAL1. Because the microarray results showed that the presence of E5 can lead to an increase in sialyltransferases expression, we evaluated the mRNA levels of ST3GAL3 and ST6GAL1 by RT-qPCR in HaCaT-E5 and HaCaT-pMSG cells. ST3GAL3 and ST6GAL1 mRNA levels were increased in HaCaT-E5 cells (Fig. 2).

We next analysed literature data for E5-modified glyco-genes that also have been previously reported as altered in cancerous tissues [Tables II and III; (11,12,22-45)]. First, we analysed the upregulated genes. As previously described, ST3GAL3 and ST6GAL1 were increased in CC and premalignant lesions; in contrast, CHST2 and MANBA have not been reported in CC, but display altered expression in other cancer types. The results showed that the four glycogenes are aberrantly expressed in several types of cancer and have clinical relevance (Table II).

We next compared the downregulated glycogenes in HaCaT-E5 cells with those reported in cancerous tissues. The four glycogenes have been reported in cancer. GALNT11 is overexpressed in chronic lymphocytic leukaemia (CLL), and UGT2B15 is downregulated in prostate cancer, but none of the downregulated glycogenes have been previously reported to be altered in CC (Table III).

E5 and glycosylation pathways. To identify possible functional associations among the enzymes identified as altered glycogenes under E5 regulation, we analysed the data with STRING software to generate predicted protein-protein interactions with a higher confidence level (0.9). For the analysis, we considered the altered glycogenes and five additional proteins with the goal of identifying possible glycosylation pathways.

For the upregulated glycogenes (ST3GAL3, CHST2, MANBA and ST6GAL1), the results displayed an interacting network with eight proteins where MANBA did not interact with any protein but participates in N-glycosylation (Fig. 3a).

Specifically, we identified the keratan sulfate biosynthesis pathway, which includes CHST2 and ST3GAL3 (Fig. 3b); the N-glycosylation pathway, in which both the sialyltransferase genes are involved in the sialylation of N-glycans (Fig. 3c); and

Figure 1. Human papillomavirus-16 E5 mRNA expression. E5 mRNA expression was 14.4 times higher in HaCaT-E5 cells compared with CasKi cells. HacaT-pMSG was used as a negative control. Data are presented as the mean ± SEM (n=3).

Table I. Sequences of the oligonucleotides used in the reverse transcription-quantitative PCR assays.

| Primer    | Forward (5'-3')             | Reverse (5'-3')             | Length (bp) |
|-----------|-----------------------------|-----------------------------|-------------|
| HPRT      | CCTGCCGTCTGTAAGTGATGAT      | CGAGCAAGACGTCTCTGTGTC       | 150         |
| HPV16 E5  | CGCTGTTTGTGCTGTGCTCT       | GCCGTCATGTGTAATAAAA         | 146         |
| ST3GAL3   | CATGTGAAGATGGGACTCTTTGG     | CCTCCCCACTGGAGTAAGTGTAG     | 118         |
| ST6GAL1   | TATCGTAGCTGCCACCCCAATC     | TTAGCAGTGAATGGTCCCGAAG     | 372         |

HPRT, hypoxanthine-guanine phosphoribosyltransferase; HPV, human papilloma virus; ST3GAL3, ST3β-galactoside α-2,3-sialyltransferase 3; ST6GAL1, β-galactoside α-2,6-sialyltransferase 1; bp, base pair.
the glycosphingolipid biosynthesis pathway, where *ST3GAL3* participates with fucosyltransferases and galactosyltransferases (Fig. 3D).

For the downregulated glycogenes (*UGT2B15, GALNT11, NDST2* and *UGT1A10*), the interaction analysis showed two different networks, among which *GALNT11* was excluded from the first network and *NDST2* establishes a separate network (Fig. 4A). The analysis showed that the latter is involved in glycosaminoglycan biosynthesis (Fig. 4B).

Discussion

Aberrant glycosylation is a characteristic of tumour cells. Changes in glycan structures in cancer have been related with altered glycogene expression (46). Increased sialylation is one of the most frequent alterations in cancer (47). Increased activity of sialyltransferases, enzymes that transfer sialic acid to glycoconjugates, has been reported during tumour transformation (11,48). Increased α2,3 and α2,6 sialic acid levels have been reported in premalignant lesions from the cervical epithelium (14). Increased expression of the sialylated antigens sLe(a) and sLe(x) have also been reported in CC and premalignant lesions (17,18). These findings could be the result of enhanced sialyltransferases genes expression that occur in the early stages of cervical cell transformation (12,13).

HPV is the aetiological agent of CC, and the HPV genome encodes three oncoproteins, E5, E6 and E7 (4); however, their roles in altering glycogene expression have been poorly investigated. Our research group is interested in the role of HPV infection and its relationship with glycogene expression changes in the cervical epithelium. Our group recently reported that the oncoproteins E6 and E7 from HPV18 modify the expression of some glycogenes, some of which participate in the glycosylation of the Notch receptor and O-glycosylation type mucin (19). However, similar reports focused on the E5 oncoprotein do not exist. E5 is a protein expressed during the early stages of viral infection, and this protein has different targets in the cell that promote cellular transformation (6,49).

With the aim of determining whether E5 modifies the expression of some glycogenes, we performed an expression microarray on the HaCaT cell line stably transfected with the HPV16 E5 oncoprotein. We identified four upregulated glycogenes (*CHST2, MANBA, ST3GAL3* and *ST6GAL1*) and four downregulated glycogenes (*UGT2B15, GALNT11, NDST2* and *UGT1A10*). All these genes have been reported to be altered in cancer either at the transcript or protein level (Tables II and III).

Increased *CHST2* mRNA levels have been reported in osteosarcoma and breast and oesophageal cancer (33-35), while increased protein levels have been reported in oesophageal cancer and CC (36). With regards to the glycogene *MANBA*, increased mRNA levels have only been reported in oesophageal cancer (37). Interestingly, of the four upregulated glycogenes, two correspond to the sialyltransferases genes *ST3GAL3* and *ST6GAL1*. *ST3GAL3* expression has been reported as altered in different cancer types, such as gastric, bile, colon, kidney, lung, breast, and ovarian cancers and glioblastoma (11,23-27). Increased *ST6GAL1* mRNA and protein expression has been reported in gastric and biliary cancers (24,27), colon and colorectal cancers (25,29,31), and ovarian and pancreatic cancers (11,30). Here, we showed that the mRNA levels of the *ST6GAL1* and *ST3GAL3* genes can be increased by the presence of E5; interestingly, both mRNA levels are increased in premalignant and malignant tissues in the cervical epithelium (12,13). However, whether these phenotypes could be a consequence of HPV infection remains unclear. Additionally, the upregulation of these genes agrees with the increased levels of sialic acid reported for CC and with the increased levels of α2,3 and α2,6 sialic acid in premalignant lesions in the cervix (14). These results suggest that the sialyltransferase expression changes that occur in the early stages of cervical cell transformation could be related to HPV infection and due to E5, but not E6 or E7, activity (19). Nevertheless, this hypothesis requires more investigation. With respect to E5 and sialylation, a previous study found no important changes in the sialylation status of keratinocytes in the presence of HPV16 E5 (50), however, in this study the authors analysed the expression of different monosaccharides and disaccharides, using a panel of seven lectins, but they did not perform glycogene expression analysis. Additionally, as they use an inducible vector, they...
Table II. Upregulated genes in HaCaT-E5 cells.

| Gene/enzyme | Enzyme function | Alteration of mRNA/enzyme in cancer | Clinical relevance |
|-------------|-----------------|------------------------------------|-------------------|
| **ST3GAL3/ST3** | Transfers sialic acid from CMP-sialic acid to the structure Galβ1→3/4GlcNAc- and is involved in the synthesis of sialyl Lewis(a) (NeuAcα2→3Galβ1→3(Fucα1→4)GlcNAc-). | Upregulation in cervical cancer (12), breast cancer (23), extrahepatic bile duct carcinoma (24), colorectal carcinoma (25), glioblastoma, renal clear cell carcinoma and lung squamous carcinoma (26). Downregulation in breast invasive carcinoma, colon adenocarcinoma (26) and ovarian cancers (11). | High levels of enzyme activity in the tumor tissue correlated with secondary local tumor recurrence in gastric cancer (27). In cervical carcinoma the overexpression of mRNA is associated with lymph node metastases (12). In breast cancer, high expression of mRNA is associated with poor prognosis (23). |
| **ST6GAL1/ST6** | Transfers sialic acid from CMP-sialic acid to the Galβ1→4GlcNAc structure on glycoproteins. | Upregulation in squamous cell carcinoma (12), breast cancer (23), extrahepatic bile duct carcinoma (24), ovarian cancer (11), and colorectal carcinoma and non-metastatic colorectal tumors (28). | Increased enzyme expression in colon tumors (28), ovarian and pancreatic carcinomas (29,30). Increased enzyme activity in gastric and colorectal cancer (27,31). In breast cancer, high expression of mRNA is associated with poor prognosis (23). In pancreatic ductal adenocarcinoma promotes chemoresistance to gemcitabine (32). |
| **CHST2/carbohydrate sulotransferase 2** | Sulotransferase that utilizes 3'-phospho-5'-adenylyl sulfate as donor to catalyze the transfer of sulfate to position 6 of non-reducing GlcNAc within keratan-like structures on N-glycans and mucin-associated glycans. | Upregulation in breast cancer (33), osteosarcoma (34) and esophageal cancer (35). | Increased enzyme expression in metastatic osteosarcoma (34). Increased enzyme in uterine cervical and corpus cancer (36). In osteosarcoma, weak protein expression is associated with improved survival (34). |
| **MANBA/β-mannosidase** | Exoglycosidase that cleaves the single β-linked mannose residue from the non-reducing end of N-glycoproteins oligosaccharides. | Upregulation in dysplastic esophageal tissues and esophageal squamous cell carcinoma (37). | No reports Candidate for molecular target for early detection of esophageal cancer (37). |

ST3GAL3, ST3 β-galactoside α2,3-sialyltransferase 3; ST6GAL1, β-galactoside α2,6-sialyltransferase 1; bp, base pair.
## Table III. Downregulated glycogenes in HaCaT-E5 cells.

| Gene/enzyme                                   | Enzyme function                                                                 | Alteration of mRNA/enzyme in cancer                                                                 | Clinical relevance                                                                 |
|-----------------------------------------------|---------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| **UGT2B15/UDP glucuronosyltransferase family 2 member B15** | An enzyme of the glucuronidation pathway, involved in the metabolism and elimination of toxic compounds. Serves a role in the regulation of estrogens and androgens. | Upregulation in castration resistant prostate cancer (38). Upregulation in gastric cancer (39). Low level of expression in aggressive prostate tumors and undetectable expression in prostate cancer with lymph node metastasis (40). | In gastric cancer, patients with higher UGT2B15 mRNA expression have poor prognosis (39). |
| **GALNT11/polypeptide N-acetylglactosaminyltransferase 11** | Catalyzes the initiation of protein O-linked glycosylation and is involved in left/right asymmetry by mediating O-glycosylation of NOTCH1. | Upregulation in chronic lymphocytic leukemia (41).                                                   | GALNT11 expression is associated with prognosis of chronic lymphocytic leukemia (41). |
| **NDST2/N-deacetylase and N-sulfotransferase 2** | Enzyme with dual functions: Participates in processing glucosamine and heparin polymers, including N-deacetylation and N-sulfation. | Moderately upregulated in hepatocellular cancer (42). Decreased levels in II, III and IV stages of glioma (43). | No reports.                                                                         |
| **UGT1A10/UDP glucuronosyltransferase family 1 member A10** | An enzyme of the glucuronidation pathway that transforms small lipophilic molecules, such as steroids, bilirubin, hormones and drugs, into water-soluble, excretable metabolites. | Upregulated in stomach cancer (44). Downregulated in breast cancer (45).                             | No reports.                                                                         |

ST3GAL3, ST3 β-galactoside α-2,3-sialyltransferase 3; ST6GAL1, β-galactoside α-2,6-sialyltransferase 1; bp, base pair.
incubated the transfected cells with dexamethasone to increase the expression of E5, so the glycosylation pattern could be influenced by the effect of this molecule, as has been reported in different studies where the dexamethasone increase the sialyltransferases expression (51,52).

Regarding the downregulated genes in the presence of HPV16 E5, GALNT11 has been reported as diminished in breast cancer (33) and increased in chronic lymphocytic leukaemia, and it was proposed to be implicated in O-glycosylation changes in chronic lymphocytic leukaemia (CLL) cells (41). NDST2 has demonstrated increased mRNA levels in hepatocellular cancer (42). It was also interesting to find expression changes in two UGT genes (UGT2B15 and UGT1A10) that encode uridine 5'-diphospho-glucuronosyltransferases as these enzymes play important roles in the biotransformation of drugs, xenobiotics, and toxic compounds (44). Expression changes in UGT genes could modify the response to some cancer drugs. UGT2B15 has been reported as diminished in prostate cancer (40), and UGT1A10 has been reported as downregulated in breast cancer (45) but upregulated in gastric cancer (44). These UGT genes have not been previously reported as altered in CC; thus, it could be interesting to analyse their expression status and
the response of patients to treatment when their expression is altered.

Additionally, we also identified possible glycosylation pathways altered in the presence of E5 by analysing protein-protein interactions. We identified keratan sulfate and glycosphingolipid synthesis as including the involvement of the glycoenzymes ST3GAL3, ST6GAL1, and CHST2. Keratan sulfate belongs to the family of glycosaminoglycans, which participates in the regulation of cellular functions in epithelial and mesenchymal tissues (53).

The glycoenzymes present in the N-glycosylation pathway are ST3GAL3 and ST6GAL1. Changes in sialic acid expression have been reported for different cancer types including CC, as was mention previously (11). Increased expression of sialylated antigens such as αLe(x) and αLe(a) has been reported in premalignant lesions and CC, and the enzyme ST3Gal III participates in the synthesis of both of these antigens (17,18,57). αLe(x) antigens can be modified by the enzyme sulfotransferase CHST2 to produce 6-sulfosialyl Lewis-x (6-sulfo sLeX), which can be a ligand of L-, P- and E-selectin (58). Moreover, ST6Gal I catalyses the α2,6 sialylation of N-glycans, and its expression is increased in different types of cancer (59). ST6Gal I had been implicated in the hypersialylation of the cell membrane protein β1 integrin in tumour cells, which leads to increased migration capacity (60). ST6Gal I also participates in the sialylation of epidermal growth factor receptor (EGFR) (61), which showed increased expression due to HPV16 E5 (62).

For the downregulated genes, the network interactions implicated in a glycosylation pathway involved only one glycoenzyme. Fig. 4 shows a network of four proteins implicated in glycosaminoglycan biosynthesis, but only one glycoenzyme, NDST2, is present in this network. The upregulation of NDST2 has been related to an increase in heparan sulfate (63).

For the glycoenzymes downregulated in the presence of E5, there are no reports focused on CC; thus, it would be important to evaluate their expression and roles in cervical transformation. The results of this study provide important information related to glycoenzymes that modify their expression in the presence of the HPV-16 E5 protein. The expression microarray generated large amounts of information on the genes that were altered, but the results must be validated. The study present some limitations, it is important to confirm that the expression of the gene is related with the expression level of its corresponding protein, additionally, the study was performed in a cell line, so the role of the HPV infection and the expression of the other viral proteins could be participating in the glycosylation changes, so the effect of E5 could be affected by other factors. Therefore, the results must be confirmed with more in vitro experiments, followed by the evaluation of glycoenzyme expression in different stages in cervical neoplasias positive for HPV16, as some of the altered glycoenzymes have not been previously reported in premalignant and malignant tissues. Also, it could be of interest to evaluate if the protein E5 from different viral genotypes, plays a different role in the glycosylation changes. Studying the effects of E5 of high and low risks HPV, will certainly provide relevant additional information of the effects of this viral infection.

The expression of the HPV16 E5 protein in HaCaT cells increased ST3GAL3 and ST6GAL1 mRNA levels, suggesting that the E5 protein could participate in the glycosylation changes found during cervical transformation in the cervical epithelium infected with HPV, especially those related to increased α2,3 and α2,6 sialylation, which have been reported as enhanced in premalignant lesions and CC. Additionally, E5 could participate in UGT gene expression changes implicated in treatment response. Changes in UGT gene expression have not been previously reported; thus, it would be interesting to analyse their levels in CC samples as well as to examine the correlation of these changes with responses to treatment.

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Availability of data and materials

The datasets generated and analysed during the current study are available in the repository NCBI Gene Expression Omnibus database, (accession no. GSE118776; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118776).

Authors’ contributions

DCR performed molecular biology experiments, in silico analysis, data analysis and revised the manuscript. YML participated in data analysis and critically revised the manuscript. PMM participated in the in silico and data analyses, and critically revised the manuscript. AAL analysed the data and critically revised the manuscript. LFJS participated in the
in silico analysis and critically revised the manuscript. GSL participated in the molecular biology experiments, analysed the data and revised the manuscript. IRL designed the current study, analysed the data and critically revised the manuscript. VVR conceived and designed the current study, coordinated the study and draft the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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