INTRODUCTION

Viruses have long been described as one of the etiologic factors in carcinogenesis. This relationship dates back about a century ago when Rous demonstrated that sarcomas can be caused by viruses. Ever since, viral oncogenesis has remained an area of interest in cancer biology. Viruses have been great tutors of cancer biology, helping researchers to uncouple many signaling pathways and identifying critical therapeutic targets. With the advent of advanced molecular techniques, more viruses have been attributed to cause neoplasms in humans. As far as the head and neck region is concerned, there is growing evidence in support of the role played by human papillomavirus (HPV) in oral squamous cell carcinoma (OSCC). Though the role of viruses in OSCC is much researched, their role in salivary gland neoplasms is not well established.

On reviewing the literature, there were not many studies that aimed to explore the role of viral oncogenesis in salivary gland neoplasms. The research on human cytomegalovirus (hCMV) was, however, quite intriguing. CMV, a member of the herpes group of viruses, has a tropism for the salivary gland ductal epithelium and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpesvirus] and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpesvirus] and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpesvirus] and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpesvirus] and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpesvirus] and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpesvirus] and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpesvirus] and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpesvirus] and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpesvirus] and establishes a persistent, lifelong infection following primary exposure. The herpes group of viruses are notorious for their striking ability to induce neoplasia [Epstein–Barr virus (EBV), Kaposi’s sarcoma herpes
Cytomegalovirus and MEC

Jayaraj, et al.

(KHSV)\textsuperscript{[3]} hCMV is also a member of the herpes group of viruses that has been implicated in malignancies of the breast, brain, lung, colon and prostate\textsuperscript{[4-12]}

Among the salivary gland neoplasms, mucoepidermoid carcinoma (MEC) is the most common malignant salivary gland neoplasm\textsuperscript{[13,14]}. Though the etiology of MEC is unknown, researchers have discovered an over-expression of COX-2 and activation of epidermal growth factor receptor (EGFR)/extracellular signal-regulated kinase (ERK) signaling pathway in MEC\textsuperscript{[13,15,16]}. Interestingly, EGFR has also been reported as a cellular receptor for CMV\textsuperscript{[17]}. The virus also results in the activation of transcription factors such as c-fos/jun, myc, NF-kB, SP-1 and mitogen-activated protein kinase (MAPK) ERK1, ERK2 and p38.\textsuperscript{[18-21]}

Therefore, considering the facts that CMV is a resident of the salivary gland duct epithelium, that it belongs to the oncogenic herpes group of viruses and employs EGFR as a portal of entry, activates pathways that are activated in MEC, there is a hypothesis that it might play a role in the pathogenesis of MEC. Though, some of the previous studies were unable to isolate the virus in salivary gland neoplasms,\textsuperscript{[22,23]} recent studies by Jaskoll \textit{et al}\textsuperscript{[24]} and Melnick \textit{et al}\textsuperscript{[25,26]} in mouse models have established that (mCMV)-induced tumorigenesis display histologic and molecular characteristics similar to human mucoepidermoid carcinoma. They also demonstrated the expression of CMV viral antigen and the upregulated COX/AREG/EGFR/ERK oncogenic signaling pathway in human MEC [Flowchart 1]. Furthermore, they were also able to demonstrate that the neoplastic phenotype induced by CMV infection was alleviated by the antivirals and small molecule inhibitors of the loop.

Therefore, with these two different schools of thoughts, the current study was designed as a pilot study to explore the possible role played by this virus in the pathogenesis of MEC in the Indian scenario where 80–90% of the adults are seropositive for CMV.\textsuperscript{[27]} Active CMV infection is associated with malignancies and pp65 antigenemia is an indication of active replication.\textsuperscript{[28]} Hence, in the study the expression of pp65 and EGFR in cases of MEC were studied. This might give an insight into the possible causal relationship between CMV and MEC.

**MATERIALS AND METHODS**

**Sample selection**

As a pilot study, the study included histopathologically confirmed cases of MEC retrieved from the archives of the department between the year 2011 and 2012. Representative cases with ample tumor tissue for immunohistochemical analysis were chosen for the study. The criteria proposed by Armed Forces Institute of Pathology\textsuperscript{[29]} was followed for grading the cases of MEC. Out of the 4 selected cases, three were low-grade MEC and one case was a high-grade MEC.

**Immunohistochemical analysis**

For the immunohistochemical study, 3 μm sections were cut from formalin fixed paraffin embedded tumor samples, mounted on gelatin coated slides. The sections were deparaffinized in xylene, dehydrated in alcohol and rinsed in distilled water. Antigen retrieval was performed using heat induced epitope retrieval in citrate buffer (pH 6.0) for 10 min in a pressure cooker. Following which endogenous peroxidase was blocked for 5 min and protein block for 5 min. Section were then incubated with anti-pp65 (NCL-CMVpp65 clone 2 and 6, Leica Microsystems, Newcastle, UK) and anti-EGFR (NCL-EGFR clone 25, Leica Microsystems, Newcastle, UK) at the manufacturer’s recommended dilution and incubated for 60 min.

Detection was performed using Novolink MinPolymer Detection System (Leica Microsystems, Newcastle, UK). The sections were deparaffinized in xylene, dehydrated in alcohol and rinsed in distilled water. Antigen retrieval was performed using heat induced epitope retrieval in citrate buffer (pH 6.0) for 10 min in a pressure cooker. Following which endogenous peroxidase was blocked for 5 min and protein block for 5 min. Section were then incubated with anti-pp65 (NCL-CMVpp65 clone 2 and 6, Leica Microsystems, Newcastle, UK) and anti-EGFR (NCL-EGFR clone 25, Leica Microsystems, Newcastle, UK) at the manufacturer’s recommended dilution and incubated for 60 min.

**Evaluation of epidermal growth factor receptor expression**

EGFR expression was evaluated using a 4-point scale classification employed by Diniz-Freitas \textit{et al}\textsuperscript{[30]} 0, no labeling
or labeling in <10% of tumor cells; 1, weak labeling in >10% of tumor cells; 2, moderate labeling in >10% of tumor cells; 3, intense labeling in >10% of tumor cells.

**Evaluation of pp65 expression**

pp65 expression was evaluated on the basis of intensity staining in tumor nucleus and cytoplasm. A 4-point scale was noted: 0 - negative staining, 1 - mild positivity, 2 - moderate positivity, 3 - severe positivity.

**RESULTS**

Normal salivary gland tissue is comprised of pale staining mucous acini and associated ducts. MEC is characterized by invasive islands of mucous and epidermoid (squamoid) cells, with or without cyst formation. Mucous cells within tumors vary in size and shape. Epidermoid cells are ovoid to polygonal with intercellular bridges and individual cell keratinization. Low-grade tumors show prominent cyst formation, predominance of mucous cells and minimal cellular atypia. High-grade tumors show little to no cyst formation, predominance of epidermoid and intermediate cells and more frequent cellular atypia [Figures 1-3].

Antibodies against pp65 determine the expression of the delayed hCMV protein in the tumors. pp65 immunostaining was absent (Grade 0) in all four cases of MEC [Figure 4] as well as in normal salivary gland tissue. The late viral antigen pp65 expression in the cases of MEC is summarized in Table 1.

EGFR expression showed a grade 2 labeling index in one case of high-grade MEC [Figure 5] but was negative “Grade 0” [Figure 6] in the other grades of MEC as well as in the normal salivary gland tissue [Figure 7]. The epidermoid cells showed positive staining for EGFR. EGFR expression distributed by histological grade of MEC is summarized in Table 2.

**DISCUSSION**

This study was designed to identify any possible causal relationship between the hCMV and MEC. The study demonstrated EGFR expression with a grade 2 labeling index in one case of high-grade MEC. The staining was

| Case number | Histological grade | pp65 expression |
|-------------|--------------------|-----------------|
| 1           | High-grade         | Negative (0)    |
| 2           | Low-grade          | Negative (0)    |
| 3           | Low-grade          | Negative (0)    |
| 4           | Low-grade          | Negative (0)    |
membranous and confined to the epidermoid cells. EGF and EGFR play an important role in growth and differentiation and also in tumorigenesis and progression of malignant disease. In this study, EGFR expression was studied along with pp65 antigen to correlate a viral induced activation of the pathway. However, considering the negative expression of pp65 antigen in the studied samples, the EGFR expression per se corroborates with that of the previous studies where high grades of MEC was associated with increased EGFR expression. Moreover, EGFR expression increased with the grade of the tumor.

Increased expression of EGFR is a marker of poor prognosis and aggressive clinical behavior. Therefore, high-grade MEC being an aggressive tumor with an infiltrative growth pattern, solid areas of epidermoid and intermediate cells, with high mitotic index and anaplasia showed an increased EGFR expression as against the low-grade tumors.

The lack of EGFR expression of low-grade MEC could be due to two reasons: (i) The negative expression might be attributed to the less aggressive nature of low-grade MEC as opposed to its high-grade counterparts. (ii) Although immunohistochemical expression of EGFR was not detected in the cases of low-grade MEC, these cases might still harbor mutations of the EGFR pathway. This discrepancy between EGFR copy number and immunohistochemical detection had been observed in several studies and could be attributed to the posttranslational modification at mRNA level.

The study could not identify the expression of the late CMV antigen in the samples of MEC. Though these results are in

Table 2: Epidermal growth factor receptor expression in the studied cases of mucoepidermoid carcinoma

| Case number | Histological grade | EGFR expression |
|-------------|--------------------|-----------------|
| 1           | High-grade         | Grade 2         |
| 2           | Low-grade          | Negative (0)    |
| 3           | Low-grade          | Negative (0)    |
| 4           | Low-grade          | Negative (0)    |

EGFR: Epidermal growth factor receptor
accordance with the works of Atula et al.,[22] Laane et al.[23] and Kärjä et al.[24] who were also unable to isolate the CMV DNA in salivary gland neoplasm samples, there needs to be a caveat in interpreting these results, especially considering the works of some researchers.[24-26] These authors through a series of studies have addressed the criteria that associate viruses and cancer. They have demonstrated that hCMV is present in most cases of human MEC and that only the neoplastic tissue harbors CMV. They have also demonstrated CMV-specific gene expression at the subcellular level and the correlation of CMV infection with an upregulated oncogenic signaling pathway. Moreover, they also demonstrated that purified CMV induces malignant transformation (MEC) in salivary gland cells in an in vitro animal model and utilizes a pathogenetic pathway similar to hCMV-induced MEC. These important findings cannot be ruled out though there are conflicting data to these results.

The lack of pp65 expression in the studied samples can be attributed to many reasons. Though the minimal sample size, the variation in the population and to methodological differences, the wider perspective here is the difficulty in isolation of CMV in human neoplasms. CMV isolation in tissues is quite challenging.[35,36] Moreover, studies have also observed that IE1 is more ubiquitously expressed in neural tissue than pp65.[37] Even in neural tumors, where CMV is believed to play an oncogenic role, some research groups were unable to identify the CMV.[38,39] Therefore, the lack of pp65 expression in the current study might also be attributed to methodological differences and the requirement of sensitive and optimized protocols to detect the very low levels of CMV that is likely to be present within the tumor. Also, in the majority of cancers caused by viral infections, the viral DNA is present in very small copy number, usually < 1 DNA copy per 10 tumor cells.[40] This might also explain the negative expression of pp65 in the studied samples.

Though, few studies[24-26] have appreciably fulfilled the stringent Koch’s Postulates, these criteria are not always fulfilled in virally induced tumors.[41] In addition to the viral agent, there are multiple factors that orchestrate carcinogenesis. Therefore, microbial genomes within cancer cells are often altered such that viable infectious pathogens are not recoverable from tumor cells.[42] Putting it altogether, the lack of viral expression in the current study might be attributed to the low copy number of the virus, the sensitivity of the technique employed, the alteration of the microbial genome and also the limited sample size.

The study was designed to test if CMV had a possible causal relationship in MEC. Though there was EGFR expression in a case of MEC, the possible viral etiology for its activation remains elusive owing to the negative expression of pp65. Though this might appear to negate the role of CMV in the pathogenesis of MEC, the challenges encountered in isolation or demonstration of the CMV viral antigen also need to be taken into consideration.

A variation in the study population is also a factor that has to be taken into consideration. Therefore, before ruling out a viral induced oncogenesis in MEC, multiple studies involving a combination of sensitized and optimized advanced molecular techniques are necessary. Yet, another avenue that could be explored to identify the viral antigen is the tumor microenvironment. In case, MEC did have a viral etiology, the tumor-associated macrophages would then express preferential viral antigens as in other CMV-induced tumors.[43]

Therefore, the main challenges in this research are improvised techniques to isolate the viral antigen from the neoplastic tissue, ability to replicate the results. This is mandatory to establish a viral etiology. This was also a major challenge even in neuro-oncology, where continued research has successfully established a viral-induced oncogenesis and development of therapeutic targets. Therefore, if proven, it would provide multiple areas of research, including the pathways involved, the viral load in the neoplastic patients, the immunosuppressive role of the virus and possibly multiple therapeutic targets that can be exploited in order to provide quality patient care and a better prognosis in these patients.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Rous P. A transmissible avian neoplasm. (sarcoma of the common fowl). J Exp Med 1910;12:696-705.
2. Nichols WG, Boeckh M. Recent advances in the therapy and prevention of CMV infections. J Clin Virol 2000;16:25-40.
3. Damania B. Oncogenic gamma-herpesviruses: Comparison of viral proteins involved in tumorigenesis. Nat Rev Microbiol 2004;2:656-68.
4. Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S, King PH, et al. Human cytomegalovirus infection and expression in human malignant glioma. Cancer Res 2002;62:3347-50.
5. Cobbs CS, Sorocauanu L, Denham S, Zhang W, Britt WJ, Pieper R, et al. Human cytomegalovirus induces cellular tyrosine kinase signaling and promotes glioma cell invasiveness. J Neurooncol 2007;85:271-80.
6. Cobbs CS, Sorocauanu L, Denham S, Zhang W, Kraus MH. Modulation of oncogenic phenotype in human glioma cells by cytomegalovirus IE1-mediated mitogenicity. Cancer Res 2008;68:724-30.
7. Giuliani L, Jaxmar T, Casadio C, Gariglio M, Manna A, D’Antonio D, et al. Detection of oncogenic viruses SV40, BKV, JCV, HCMV, HPV and p53 codon 72 polymorphism in lung carcinoma. Lung Cancer 2007;57:273-81.
8. Harkins L, Volk AL, Samanta M, Mikolaenko I, Britt WJ, Bland KI, et al. Specific localisation of human cytomegalovirus...
nucleic acids and proteins in human colorectal cancer. Lancet 2002;360:1557-63.
9. Harkins LE, Matlaf LA, Soroceanu L, Klemm K, Britt WJ, Wang W, et al. Detection of human cytomegalovirus in normal and neoplastic breast epithelium. Herpesviridae 2010;1:8.
10. Lucas KG, Bao L, Bruggeman R, Dunham K, Specht C. The detection of CMV pp65 and IE1 in glioblastoma multiforme. J Neurooncol 2011;103:231-8.
11. Samanta M, Harkins L, Klemm K, Britt WJ, Cobbs CS. High prevalence of human cytomegalovirus in prostatic intraepithelial neoplasia and prostatic carcinoma. J Urol 2003;170:998-1002.
12. Scheuer ME, Bondy ML, Aldape KD, Albrecht T, El-Zein R. Detection of human cytomegalovirus in different histological types of gliomas. Acta Neuropathol 2008;116:79-86.
13. Lujan B, Hakim S, Moyano S, Nadal A, Caballero M, Diaz A, et al. Activation of the EGFR/ERK pathway in high-grade mucoepidermoid carcinomas of the salivary glands. Br J Cancer 2010;103:510-6.
14. Schwarz S, Stiegler C, Müller M, Ettl T, Brockhoff G, Zenk J, et al. Salivary gland mucoepidermoid carcinoma is a clinically, morphologically and genetically heterogeneous entity: A clinicopathological study of 40 cases with emphasis on grading, histological variants and presence of the t(11;19) translocation. Histopathology 2011;58:557-70.
15. Akrish S, Peled M, Ben-Izhak O, Nagler RM. Malignant salivary gland tumors and cyclo-oxygenase-2: A histopathological and immunohistochemical analysis with implications on histogenesis. Oral Oncol 2009;45:1044-50.
16. Ito FA, Ito K, Coletta RD, Graner E, de Almeida OP, Lopes MA. Salivary gland tumors: Immunohistochemical study of EGF, EGFR, ErbB-2, FAS and Ki-67. Anal Quant Cytol Histol 2009;31:280-7.
17. Wang X, Huang SM, Chiu ML, Raab-Traub N, Huang ES. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature 2003;424:456-61.
18. Kowalik TF, Wing B, Haskill JS, Azizkhan JC, Baldwin AS Jr., Huang ES. Multiple mechanisms are implicated in the regulation of NF-kappa B activity during human cytomegalovirus infection. Proc Natl Acad Sci U S A 1993;90:1107-11.
19. Yurochko AD, Kowalik TF, Huang SM, Huang ES. Human cytomegalovirus upregulates NF-kappa B activity by transactivating the NF-kappa B p105/p50 and p65 promoters. J Virol 1995;69:5391-400.
20. Boyle KA, Pietropaolo RL, Compton T. Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon-responsive pathway. Mol Cell Biol 1999;19:3607-13.
21. Boldogh I, AbuBakar S, Deng CZ, Albrecht T. Transcriptional activation of cellular oncogenes fos, jun, and myc by human cytomegalovirus. J Virol 1991;65:1568-71.
22. Atula T, Grènman R, Klemi P, Syrjänen S. Human papillomavirus, Epstein-Barr virus, human herpesvirus 8 and human cytomegalovirus involvement in salivary gland tumours. Oral Oncol 1998;34:391-5.
23. Laane CJ, Murr AH, Mhatre AN, Jones KD, Laiwani AK. Role of Epstein-Barr virus and cytomegalovirus in the etiology of benign parotid tumors. Head Neck 2002;24:443-50.
24. Jaskoll T, Htet K, Abichaker G, Kaye FJ, Melnick M. CRTCl expression during normal and abnormal salivary gland development supports a precursor cell origin for mucoepidermoid cancer. Gene Expr Patterns 2011;11:57-63.
25. Melnick M, Mocarski ES, Abichaker G, Huang J, Jaskoll T. Cytomegalovirus-induced embryopathy: Mouse submandibular salivary gland epithelial-mesenchymal ontogeny as a model. BMC Dev Biol 2006;6:42.
26. Melnick M, Sedghizadeh PP, Allen CM, Jaskoll T. Human cytomegalovirus and mucoepidermoid carcinoma of salivary glands: Cell-specific localization of active viral and oncogenic signaling proteins is confirmatory of a causal relationship. Exp Mol Pathol 2012;92:118-25.
27. Boppana SB, Fowler KB. Persistence in the population: Epidemiology and transmission. In: Arvin A, Campadelli-Fiume G, Mocarski ES, Moore PS, Roizman B, Whitley R, et al., editors. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press; 2007.
28. van den Berg AP, van der Bij W, van Son WJ, Anema J, van der Giessen M, Schirm J, et al. Cytomegalovirus antigenemia as a useful marker of symptomatic cytomegalovirus infection after renal transplantation – A report of 130 consecutive patients. Transplantation 1989;48:991-5.
29. Goode RK, Auclair PL, Ellis GL. Mucoepidermoid carcinoma of the major salivary glands: Clinical and histopathologic analysis of 234 cases with evaluation of grading criteria. Cancer 1998 1;82:1217-24.
30. Diniz-Freitas M, García-Caballero T, Antúnez-López J, Gándara-Rey JM, García-García A. Pharmacodiagnosis evaluation of EGFR expression in oral squamous cell carcinoma. Oral Dis 2007;13:285-90.
31. Arteaga C. Targeting HER1/EGFR: A molecular approach to cancer therapy. Semin Oncol 2003;30 3 Suppl 7:3-14.
32. Monteiro LS, Bento MJ, Palmeira C, Lopes C. Epidermal growth factor receptor immunoexpression evaluation in malignant salivary gland tumours. J Oral Pathol Med 2009;38:508-13.
33. Grandis JR, Twardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 1993;53:3579-84.
34. Kärjä V, Syrjänen K, Syrjänen S. No Epstein Barr and cytomegalovirus DNA found in salivary gland tumours. Oral Oncol 1998;34:391-5.
35. Sabatier J, Uro-Coste E, Labrousse F, Allart S, Trémoulet M, et al. Detection of human cytomegalovirus genome and gene products in central nervous system tumours. Br J Cancer 2005;92:747-50.
36. Poltermann S, Schlehofer B, Steindorf K, Schnitzler P, Geletneky K, Schlehofer JR. Lack of association of herpesviruses with brain tumors. J Neurovirol 2006;12:90-9.
37. Mitchell DA, Xie W, Schmittinger R, Learn C, Friedman A, McLendon RE, et al. Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. Neuro Oncol 2008;10:10-8.
38. Lau SK, Chen YY, Chen WG, Diamond DJ, Mamelak AN, Zaia JA, et al. Lack of association of cytomegalovirus with human brain tumors. Mod Pathol 2005;18:838-43.
39. Duncan CG, Leary RJ, Lin JC, Cummins J, Di C, Schaeler CF, et al. Identification of microbial DNA in human cancer. BMC Med Genomics 2009;2:22.
40. Sampson JH, Mitchell DA. Is cytomegalovirus a therapeutic target in glioblastoma? Clin Cancer Res 2011;17:4619-21.
41. Zur Hausen H. The search for infectious causes of human cancers: Where and why. Virology 2009;392:1-10.
42. Dziurzynski K, Wei J, Qiao W, Hattiboglu MA, Kong LY, Wu A, et al. Glioma-associated cytomegalovirus mediates subversion of the monocyte lineage to a tumor propagating phenotype. Clin Cancer Res 2011;17:4642-9.