Veterinary Microbiology 195 (2016) 96–100

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Felis catus papillomavirus type 2 E6 oncogene enhances mitogen-activated protein kinases and Akt activation but not EGFR expression in an *in vitro* feline model of viral pathogenesis

Gennaro Altamura[a], Annunziata Corteggio[b], Giuseppe Borzacchiello[a,*]

[a] Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Via F. Delpino, 1, 80137 Napoli, Italy
[b] Institute of Protein Biochemistry (IBP), National Council of Research (CNR), Via Pietro Castellino, 111, 80131 Napoli, Italy

ARTICLE INFO

Article history:
Received 23 May 2016
Received in revised form 13 September 2016
Accepted 20 September 2016

Keywords:
EGFR
FcaPV2
Feline SCC
MAPK
pAkt

ABSTRACT

A possible causative role of *Felis catus* papillomavirus type 2 (FcaPV2) in the development of feline oral and cutaneous squamous cell carcinomas (SCC) has been recently suggested by demonstrating viral gene expression *in vivo* and transforming properties by its putative oncogenes E6 and E7 *in vitro*. The activated molecules MEK (pMEK), ERK (pERK) and Akt (pAkt) are signaling transduction effectors regulating cell proliferation and inhibition of apoptosis, which are critical steps towards tumour formation. Here, we show by Western blotting (WB) that expression of FcaPV2 E6 in feline epithelial cells enhances pMEK, pERK and pAkt levels compared to control cells. Additionally, we demonstrated by real-time quantitative PCR on epidermal growth factor receptor (EGFR) transcripts and WB that activation of these signaling routes is independent from EGFR differential gene expression, total protein levels or phosphorylation, unlike in human papillomavirus associated tumours. This study contributes to define the molecular scenario underlying FcaPV2-triggered pathogenesis of feline SCC.

1. Introduction

Feline squamous cell carcinomas (SCC) are epithelial tumours of skin and oral cavity of cats which are often associated with DNA of *Felis catus* papillomavirus type 2 (FcaPV2) (Munday, 2014; Altamura et al., 2016). Like other oncogenic papillomaviruses (PVs), FcaPV2 genome harbors E6 and E7 putative oncogenes, however functional studies regarding the virus-triggered pathogenesis of these lesions are lacking (Lange et al., 2009). Recently, we have demonstrated FcaPV2 genes expression in oral and skin SCCs *in vivo* and the transforming abilities by E6 and E7 oncoproteins in corrupting p53 and pRb pathways *in vitro*, suggesting a possible causative role of this virus in the development of feline SCC (Altamura et al., 2016).

The mitogen-activated protein kinases (MAPK) MEK and its downstream effector ERK are key molecules of the Ras-MAPK signaling route which is known to regulate cellular proliferation; Akt is a serine-threonine kinase involved in the phosphatidyl inositol 3-kinase pathway (PI3K) which plays a role in inhibition of apoptosis (Ranieri et al., 2013). MEK, ERK and Akt are activated by phosphorylation (pMEK, pERK and pAkt) and they are downstream effectors of the membrane tyrosine kinase receptors (TKRs), which are activated by binding of their specific ligands represented by growth factors (Ranieri et al., 2013). Once TKRs activity is dysregulated, their signaling transduction pathways may contribute to the development of cancer in humans as well as in animal species (Ranieri et al., 2013). The epidermal growth factor receptor (EGFR) is a TKR known to be involved in different kinds of tumours including human PVs (HPVs) induced cancers, where it is overexpressed and/or hyperactivated upon HPVs oncogenes expression (Hu et al., 1997; Spangle and Munger, 2013; Simpson et al., 2015). Interestingly, expression of EGFR has been reported also in feline oral and skin SCC and inhibition of EGFR pathway through small inhibitor molecules has been proposed as therapeutic tool for this severe and often lethal cancer of domestic cat (Sabattini et al., 2010; Bergkvist et al., 2011). The aim of this study was to assess whether expression of FcaPV2 oncogenes may cause the activation of MAPK and Akt in feline epithelial cells and whether this may be dependent upon EGFR dysregulation as in the human counterpart.

* Corresponding author.
E-mail addresses: gennaro.altamura@unina.it (G. Altamura), a.corteggio@ibp.cnr.it (A. Corteggio), borzacch@unina.it (G. Borzacchiello).

http://dx.doi.org/10.1016/j.vetmic.2016.09.013
0378-1135/

2. Materials and methods

2.1. Cells and cell culture

Crandel-Rees feline kidney (CRFK) and HeLa cells were purchased from ATCC cell bank. CRFK expressing FcaPV2 genes E6E7, E6 and E7 cloned in pCEFL vector had been generated in our laboratory using standard cloning and transfection procedures and expression of viral genes previously demonstrated (Altamura et al., 2016). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 ug/mL streptomycin (Gibco). For starvation experiments, 70–80% confluent cell monolayers were kept in DMEM without FBS for 24 h. All the cell lines were grown at 37 °C in humidified atmosphere at 5% CO_2.

2.2. Western blotting and antibodies

For Western blotting analysis, 70–80% confluent cell monolayers in 60 mm Petri dishes were washed in phosphate-buffered saline (PBS) and then lysed in ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.25% Deoxicholate) added with protease and tyrosine and serine-threonine phosphatase inhibitors cocktails (10 uL for each mL, Sigma), for 20 min on ice. Cell lysates were clarified by centrifugation at 13000 rpm for 10 min at 4 °C. Protein concentration was measured by Bradford protein assay (Bio-Rad). Equal amounts of each protein sample were mixed to Laemmli sample buffer (sodiumdodecyl sulphate (SDS), Tris-HCl pH 6.8%, glycerol, bromophenol blue, and β-mercaptoethanol), boiled and loaded onto polyacrylammide gels to be subjected to separation by electrophoresis. Proteins were transferred onto nitrocellulose membranes using TransBlot Turbo apparatus (Bio-Rad). Membranes were incubated with 5% Non Fat Dry Milk (NFDM) in Tris Buffered Saline (TBS) 0.1% Tween-20 (TBST) for 1 h at room temperature, to block non specific bindings, and then primary antibodies diluted in TBST 5% NFDM were applied over night at 4 °C with gentle agitation. After washing steps in TBST, membranes were incubated with appropriate secondary antibodies conjugated with Horseradish Peroxidase (HRP) (GE Healthcare) for 1 h at room temperature. Following further washings in TBST, bound antibodies were visualized by enhanced chemiluminescence (ECL, Clarity ECL Western Blotting Substrate, Bio-Rad). Protein levels were quantitatively estimated by densitometric analysis using ChemiDoc gel scanner (Bio-Rad) equipped with a densitometric workstation (Image Lab software, Bio-Rad).

The following primary antibodies were purchased from Santa Cruz Biotechnology: MEK (sc-436), pMEK (sc-7995-R), ERK (sc-135900), pERK (sc-16982-R), Akt (sc-1619), pAkt (7985-R), pEGFRtyr1173 (sc-12351-R), pEGFRtyr1068 (sc-81488). Anti-EGFR antibody was from Thermo Scientific (MS-400-P0), anti-β-actin antibody was from Calbiochem (CP01). The reactivity of the antibodies was ensured by running HeLa whole cell lysates along with feline cells samples.

Protein expression levels were normalized to β-actin; pMEK, pERK and pAkt were normalized to total MEK, ERK and Akt, respectively.

2.3. RNA extraction, reverse transcription (RT) and real time-quantitative PCR (qPCR)

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) following the manufacturer recommendations. RT was performed on 500 ng of total RNA using iScript cDNA Synthesis Kit (Bio-Rad); 25 ng of cDNA were subjected to real-time qPCR for feline EGFR using iTaq Universal SYBR Green Supermix according to the brand instructions (Bio-Rad). Amplification of feline GAPDH was performed in parallel to allow normalization of the results. The following primers pairs were used: EGFR-Fw:

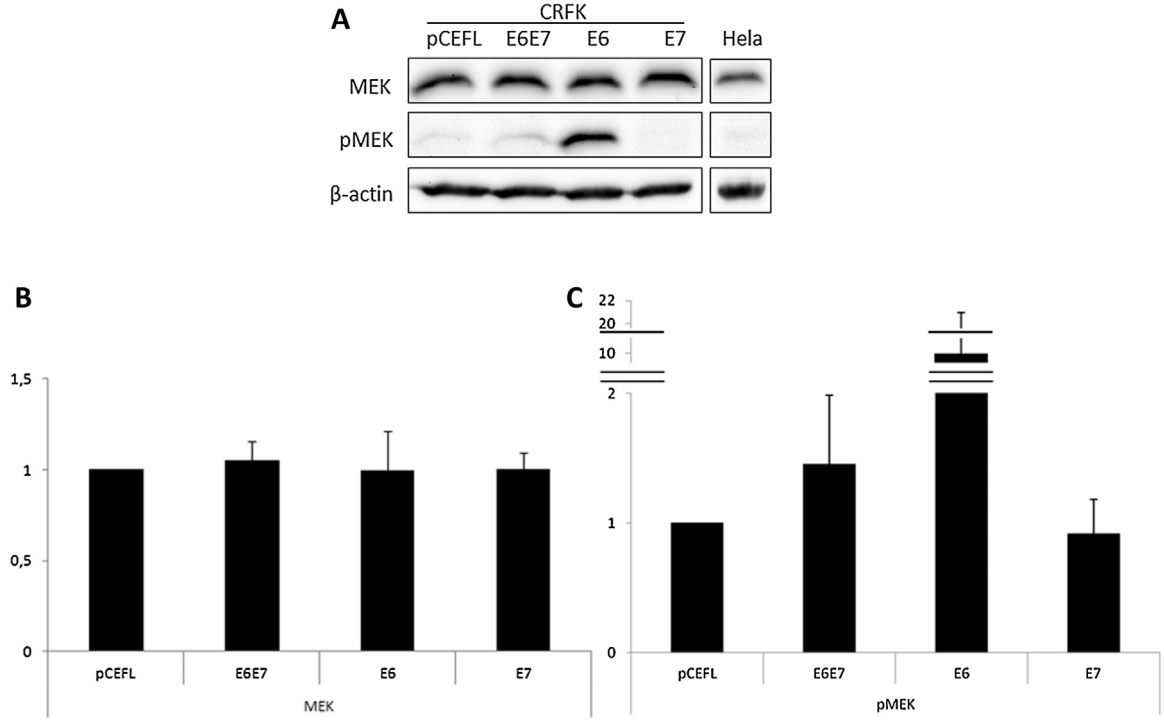

Fig. 1. (A) Representative WB analysis of MEK and pMEK in CRFK cells expressing FcaPV2 E6E7, E6 and E7. The blot was stripped and reprobed with anti-β-actin antibody to confirm equal loading of proteins in each lane. (B, C) Mean densitometric values and SD from independent experiments showing equal amounts of MEK but upregulation of pMEK (normalized to total MEK) in CRFK E6E7 and E6 compared to CRFK pCEFL (empty vector) (the data are expressed as fold change).