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HPV18 E6 and E7 Intratumour Heterogeneity in Esophageal Cancer

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Abstract

The development of esophageal cancer accompanied by the presence of human papillomavirus (HPV) DNA into the host genome. By evaluating the expression of this virus for tumor cell origin and also their cell grows and migrations, we examined esophageal cancer clonality in the context of intra-tumor heterogeneity. In this research, we have checked the expression of HPV18 E6 and E7 in different single cell clones by the manual cell picking method in the HPV positive esophageal cancer (EC109), EC109 cell line used as a negative control, and Hela cell line used as the positive control. Quantitative real-time PCR (QRT-PCR) was run to detect the expression levels of HPV E6 and E7, Cell Counting Kit-8 (CCK-8) assay was used to examine cell proliferation, invasion assays performed using Costar chambers and wounding assay to study cell migrations *in vitro*. We investigated the intra-tumor heterogeneity of HPV E6 and E7 in esophageal cancer and the evaluation of the growth and migrations at the clonal level, using 10 single cell clones. In particular clones, C7 & C10 displayed a highly variable expression in both HPV E6 and E7 and weak in four clones (C1, C3, C4, and C9) consequently, the cell invasion, proliferation, and migration increase with increasing the level of HPV expression and inverse. In conclusion, the resulting based on single cell cloning showed the relationship between HPV and cell growth and migration in esophageal cancer. Future study in HPV DNA integration needed to explore the mains specific integration site of HPV DNA in esophageal cancer and molecular monitoring of the HPV for future prevention researches and also effective therapeutic strategies.

#The two authors contribute equally.

Keywords

Esophageal Cancer, Human Papillomavirus, HPV 18 E6 and E7, Single Cell Cloning, Intra-Tumor Heterogeneity

1. Introduction

The population of cancer cells have detected in different researches and explained heterogeneity in term of tumorigenicity, mutations, activation of metabolic and signaling pathway, grows and migrations, different ploidy, metastasis, alternation of copy number, and responding to the anticancer agent [1] [2] [3]. Cell heterogeneity can be observed in the different group of patients and also different tumors in the same organ (inter-tumor heterogeneity) and different cells in the same tumor (intra-heterogeneity) [4] [5] [6].

The idea of the intera-heterogeneity is back to at least to the 1970s when the same tumor in the mouse models had different sensitivities to cytotoxic therapy or different tendencies to metastasize [7].

Tumor heterogeneity has observed in different kind of cancers like esophageal cell carcinoma [8]. Afterward, studies detected the HPV DNA in esophageal cancer base on intra-heterogeneity. Research evidence that, the integration of HPV DNA may result in esophageal cancer intra-heterogeneity. Esophageal cancer is following to steady infection of esophagus epithelial cells with oncogenic types of HPVs [9]. Early carcinogenesis described by constant integration of HPV DNA into the host genome [10]. Following, rising HPV DNA integrity results in increasing HPV DNA expressions and consequently, cell grows and proliferation [11].

However, the HPV DNA contribution in the context of single clones has not widely studied. In this research we are going to proof intra-tumour heterogeneity in esophageal cancer by detecting HPV E6 and E7 genes in 10 different single clones of esophageal cancer cells and clonal expansion by evaluating proliferation as well as invasions and migrations of each single clone to better understand HPV E6 and E7 roles in ESCC intra-heterogeneity and disease monitoring.

2. Materials and Methods

2.1. Cell Lines and Single Cell Clones

The esophageal squamous cell carcinoma cell lines (EC109) were obtained from the Zeng Academician Laboratory of the Virus Prevention and Control Institute (CDC, Beijing, China). All cells were cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (10% FBS), and they were seeded in 96-well plates at a calculated 1 cell/well in each test medium, and maintained in a humidified incubator at 37˚C with 5% CO_2.

2.2. RNA Extraction and qRT-PCR

We used Trizol reagent (Takara, Japan) to isolate the Total RNA from culture's

cell, and then using the Prime Script RT reagent Kit (Promega, USA) to synthesize the cDNA from a total of 400 ng RNA according to the manufacturer's protocol. cDNA amplified by quantitative real-time PCR with SYBR Green Kit (Promega, USA). For normalizing the level of HPV18 E6 & E7 we used GAPDH. Primers for HPV 18E6, E7 and GAPDH are listed in Additional **Table 1**.

2.3. Cell Proliferation Assay

Cell proliferation was performed every 12 hours in 96-well plates and 1000 cells per wells by using cell counting kit8 (Djingo, Japan) as the manufacturer's protocol.

2.4. Invasion Assays

Single clone cells seeded (5×10^4) were suspended in 200 µl of serum-free medium and seeded in the upper Costar chambers containing transwell inserts with a pore size of 8 µm (Corning Incorporated, USA), and coated with Matrigel (Invitrogen, USA). While the bottom chamber contained medium mixed with 20% FBS. We were fixed the cells with methanol and stained with 0.1% crystal violet, then removing the extra cells by using cotton swap in the upper chamber after forty-two hours. Then imaged, and counted under an inverted microscope in three random fields (Olympus, Japan).

2.5. Wounding Assay

The wound was made on the monolayer cells and capturing. Cells started to migrate at the wound edges then we have captured the images as the second stages of our experiment after 12 hours. Then we have calculated the migrations speed of the cells in certain clones by comparing these two stages together.

2.6. Statistical Analysis

The statistical significance analysis was examined at the mean ± standard deviation (SD), using T-test and ANOVA. All analyses were performed on SPSS 21.0, and ($P < 0.05$) was considered significant.

3. Results

3.1. Esophageal Cancer Single Clones and RT-PCR Analysis of HPV E6 and E7 Expression

To analyze the expression of HPV18 E6 and E7 at the clonal level, 10 single cell clones were obtained from a primary culture of the human esophageal cancer (EC109). In this case, we used manual cell picking metode. The cells are typically provided as a suspension in a 96 well-plate. The single-seeded cell was choosing via microscope observation. And obtained clones from these single cells were cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (10%FBS), and maintained in a humidified incubator at 37˚C with5% CO_2. These clones were evaluated by the Qrt-PCR to detect the expression level of

Table 1. Primers for HPV E6 and E718 E6, E7 and GAPDH

	SENSE(5'-3')	ANTISENSE(5'-3')
GAPDH	ACCACAgTCCATgCCATCAC	TCCACCACCCTgTTgCTgTA
E718 E6	ggTgCCAgAAACCgTTgA	TgCgTCgTTggAgTCgT
HPV18 E7	TAAgCgACTCAgAggAAgAA	gCTggAATgCTCgAAgg

HPV18 E6 and E7 fragments. Results revealed that a heterogeneous expression of HPV E6 and E7 in investigated clones (**Figure 1**) and significant difference between them ($P < 0.05$). According to these results, particular clones displayed a highly variable expression in both HPV E6 and E7 (C7, C8 and C10) had low expression in clones C3, C4, and C5.

3.2. Cell proliferation in Different Single Clones

We were used CCK-8 assays to detect the effect of intra-heterogeneity on cell proliferation and growth as shown in (**Figure 2**). Significant difference was observed between clones in proliferation ($P < 0.05$). Our results demonstrated those clones which have high expression of HPV 18 E6 and E7 significantly promoted cell proliferation.

3.3. Single Clone Cells Migration and Invasion

To detect the functions of HPV18 E6 and E7 in esophageal cancer migration and invasion, we conduct the wounding and Tran swell chamber assays. The significant difference was observed between clones in both wounding and invasion assays ($P < 0.001$). Single clones which had high expression of HPV 18 E6 and E7 significantly promoted cell migration through a permeable filter and invasion through Matrigel Matrix (**Figure 3** and **Figure 4**).

4. Discussions

One of the main reasons for poor effective therapies in esophageal cancer is an inappropriate prognosis [12]. Study on cancer cell heterogeneity is required for detecting the appropriate molecular prognostic markers as well as patients' classifications and using them for specific and effective therapies. Afterward, research efforts for heterogeneity descriptions would be helpful for better understanding of the disease progressing and diagnosis [11].

Heterogeneity mentioned the different morphology and genotype in distinct tumor cells, this phenomenon can happen between tumors and also within tumors which is called inter-tumor heterogeneity and intra-tumor heterogeneity respectively [4] and caused by both genetic and non-genetic factors in a variety of cancers as well as esophageal cancer [13].

HPV E6 and E7 in esophageal cancer have detected [14], and the role of these oncogenes in heterogeneity in plenty of researches evaluated.

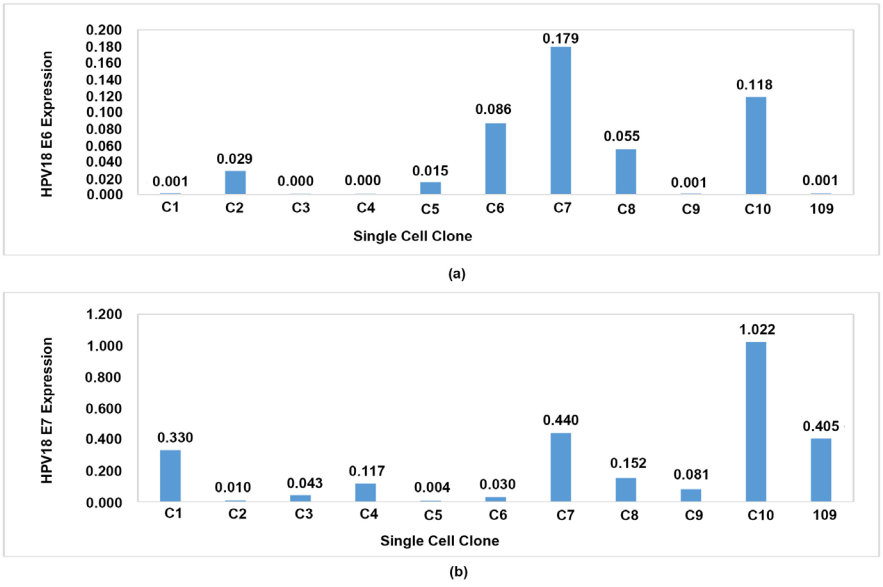

Figure 1. QRT-PCR analysis performed to evaluate the expression of HPV 18 E6 (a) and E7 (b) in 10 single clones of HPV positive esophageal cancer (EC109), EC109 as a negative control and Hela cell line used as the positive control. Significantly difference observed between different clones ($P < 0.05$).

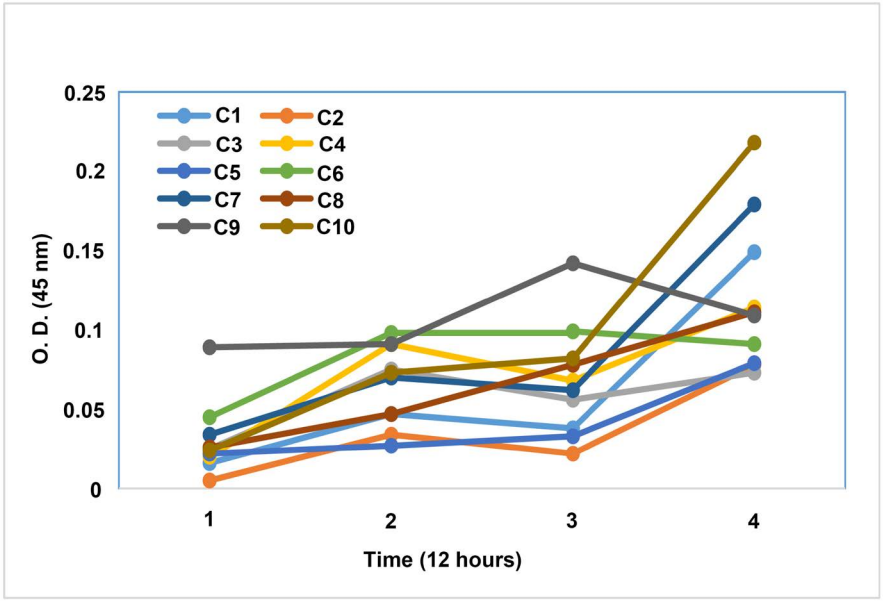

Figure 2. The proliferation assay performed, using CCK-8 kit every 12 hours and results of 10 single Cell clones and EC109 celline, considering a significant difference between clones ($P < 0.05$).

The studies demonstrated that the different HPV gene expressions in each single clones. It is may because of HPV DNA can exist in tow form in human genome, integrate or non integrated. Upon infection, firstly HPV genome amplified as episomes in the cell then some of them subsequently integrate randomly in to the host genome in one or more different location. Research showed

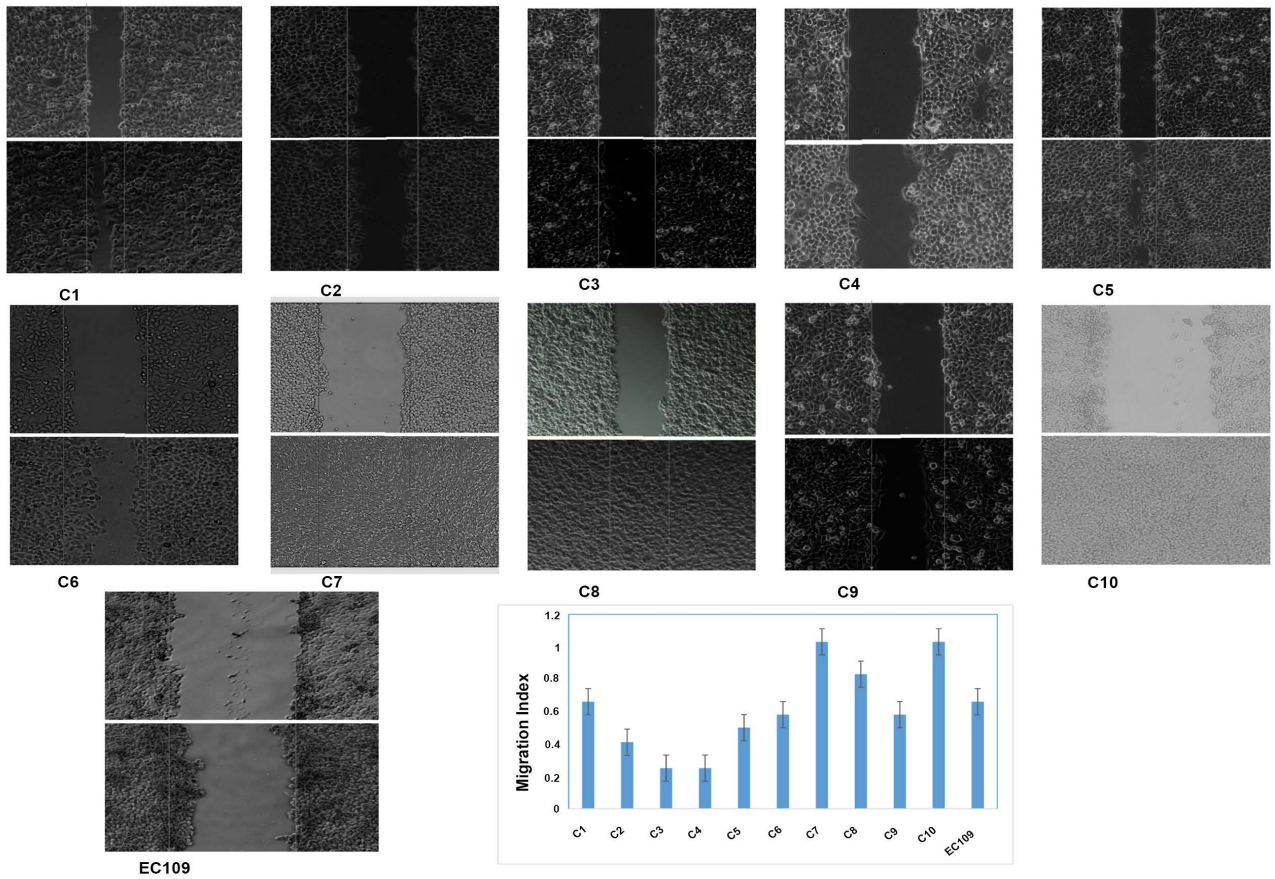

Figure 3. Wound healing assay performed in 10 different single clones of EC109 cellines. the wounds were captured pictures at 0 and 12 hours post-scratch (10× magnifications) and the results calculated at the two time points in different single clones as well as control cell lines. Significantly difference was observed between cell lines ($P < 0.001$).

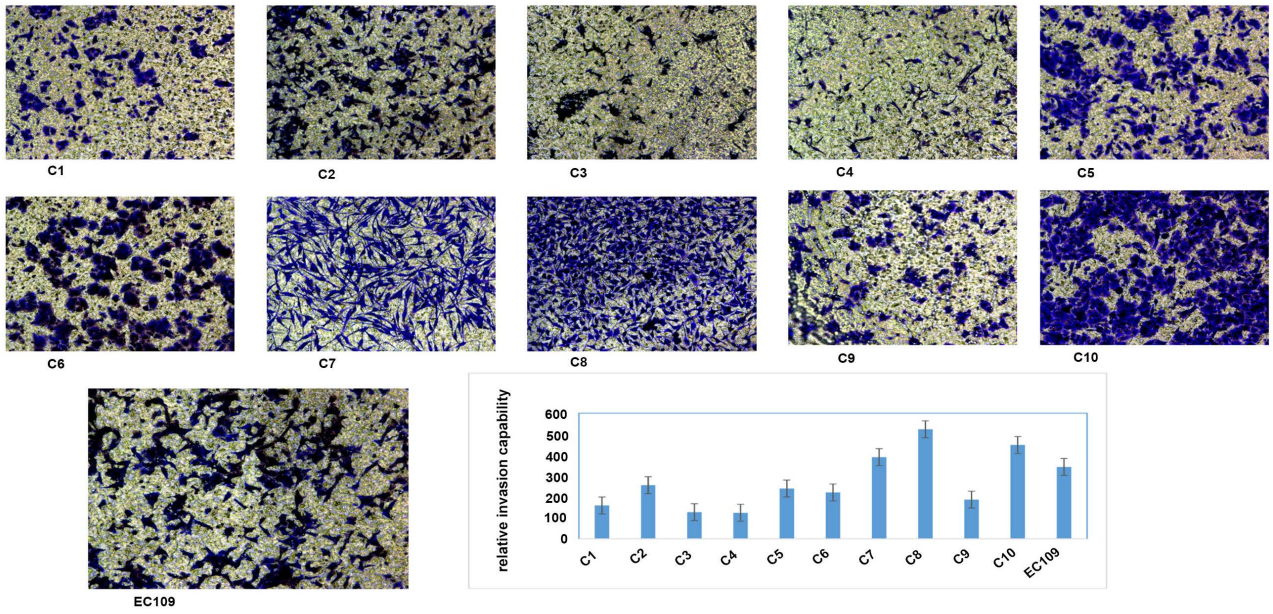

Figure 4. Invasion assay performed in 10 single clones of EC109 cell line, EC109 and Hela used as a negative and positive control controls, using a transwell system. And the cells were captured in 10× magnifications. Significantly invasion observed between different single clones ($P < 0.001$).

that there is strongly associated between HPV integrate DNA and proliferation increasing in compare with episomal HPV DNA [11].

In this study 10 single clone HPV 18 positive esophageal cancers were obtained and the expression levels of E6 and E7 oncogenes were detected indifferent single clones. The results showed the heterogeneous expression of E6 and E7. Some clones explaned highly expressed of both E6 and E7 (7C, C8 and C10) and some had low expression of these tow oncogenes (C3, C4 and C5). As far as Human papillomavirus 16/18 promotes cancer cell proliferation, migration and invasion [15], this study was conducted to check the cell cycle regulations of each single clone and result demonstrate that those single clone cells with high expression of HPV E6 and E7 had high level of proliferation, migrations and also invasion.

5. Conclusion

This research revealed that the role of HPV 18 E6 and E7 in cell regulations in the context of intra-heterogeneity in ESCC. It may potentially help to monitor and progress of HPV positive esophageal cancer therapies and diagnostic.

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Availability of Data and Materials

All available data can be obtained by contacting the corresponding author.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Patient Consent for Publication

Patient consent was obtained from all individuals in this study.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Manuscript Materials

The manuscript contains original material. And the content has not been published or submitted for publication elsewhere.

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