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Epigallocatechin gallate, folic acid, vitamin B12, and hyaluronic acid significantly increase apoptosis and p53 expression in HeLa cells

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Abstract. – OBJECTIVE: The human papilloma virus (HPV) is the etiological agent of cervical cancer in more than 95% of cases worldwide. Although most HPV infections clear up on their own and most pre-cancerous lesions spontaneously resolve, in some cases, they can persist, leading to lesions which may progress towards invasive cervical cancer.

MATERIALS AND METHODS: We evaluated the effects of the association of epigallocatechin gallate (EGCG) + folic acid (FA) + vitamin B12 (B12) + hyaluronic acid (HA) on HPV-positive cervical cancer cells (HeLa).

RESULTS: The association of EGCG + FA + B12 + HA induced a significant increase of apoptosis and p53 gene expression with a concomitant decrease of E6/E7 gene expression, a marker of HPV infection.

CONCLUSIONS: This study provides for the first-time evidence on the potential additive activity of EGCG + FA + B12 + HA in counteracting HPV infection, by increasing apoptosis and p53 expression in HPV-infected cervical HeLa cells.

Key Words:
Cervical cancer, p53, Apoptosis, E6/E7, Epigallocatechin gallate, Folic acid, Vitamin B12, Hyaluronic acid, Additive effect.

Introduction

Human papillomavirus (HPV) infection strictly correlates with the development of more than 95% of cervical cancer, the second most common form of cancer among young women between 15 and 45 years of age[1]. To date, 200 different types of HPV have been identified and classified into several phylogenetic groups according to their oncogenicity. Among them, the most frequently detected HPV type at the time of diagnosis of squamous cell carcinoma (SCC) is HPV16, followed by HPV18. This latter is the type most strongly associated with adenocarcinoma of the cervix, whose incidence is increasing[2].

Despite most genital HPV infections spontaneously resolve within two years of infection, persistence occurs in about 20% of cases, leading to increased risk of developing precancerous lesions and invasive cervical cancer within 10-20 years[3]. Persistence can lead to the integration of HPV DNA in host genome. This event specifically interferes with the function of the E2 protein, which normally downregulates the transcription of E6 and E7 viral genes and leads to an increased expression of E6 and E7 oncoviral proteins[4]. These two viral oncoproteins contribute to maintain the HPV infection by abrogating Rb and p53 functions, respectively, which are two tumor suppressor genes responsible for most of the cells' antitumor mechanisms[5]. It is worth noting that programs of primary and secondary prevention (i.e., vaccines and periodic screening, respectively) are the only available tools against the HPV infection. Current therapies may only target clinical signs of the infection, but no specific approach exists against the virus, much less against its persistence[6]. Therefore, during the past decades, the research focused on the cancer chemo-preventive activity of different natural molecules, to fill the still-existing gap in clinical practice[7]. Among them, epigallocatechin gallate (EGCG) extracted from green tea[8] has anti-proliferative and pro-apoptotic activities and can stimulate the expression of the

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pro-apoptotic marker p53 by reducing the levels of viral oncoproteins E6/E7[9]. In addition, vitamin B12 (B12) and folic acid (FA)[10] seem to be involved in the methylation of the HPV genome, thus contributing to blocking viral proliferation, and increasing the clearance of the virus. Clinical evidence revealed that high levels of both micronutrients may reduce the risk of tumoral progression for the high-grade cervical lesions associated with HPV infections. Moreover, high levels of FA have a protective effect against HPV transmission, as they correlate with a 73% lower probability of infection[11].

HPV is transmitted through direct or indirect contact with an individual who has already the infection, preferably in the affected areas during sexual intercourse. Dysfunctions in the epithelial barrier due to trauma and/or minor injuries cause loss of continuity, and microtrauma in the skin thus allowing viral particles penetration and infection[12]. Therefore, preventing HPV infection could be the goal to maintain or restore the integrity of cervical tissue. Hyaluronic acid (HA), a component of the extracellular matrix (ECM), can be a valid aid in this regard. HA has several functions, which depend on its molecular weight. In particular, very low molecular weight HA (<5 kDa), promotes the process of wound-healing repair by stimulating the production of pro-inflammatory factors[13,14]. Therefore, the reparative action of HA could play a crucial protective role as a physical barrier to block HPV entry by restoring the integrity of the damaged epithelium or mucosae.

Before testing these HA properties, we first tested its security profile in association with other natural molecules.

In fact, despite this scientific evidence, the effects of the combination of EGCG, FA, vitamin B12, and HA have no proper scientific investigation yet. Therefore, the goal of this research was to assess whether these natural molecules together could affect markers of the HPV infection and persistence with its consequent tumorigenic induction.

Materials and Methods

Cell Culture

The human HeLa cervical carcinoma cell line was obtained from European Collection of Cell Cultures (ECACC). The cells were seeded into 25 cm^2 flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and grown in monolayer culture in a 1:1 mixture of Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) and Ham's F12 medium containing 5% fetal bovine serum (FBS), antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL, gentamycin 200 µg/mL; all from Euroclone Ltd, Cramlington, UK). The cells were cultured at 37°C in air with 5% CO^2. The medium was changed every three days. At confluence, the cells were subcultured after removal with 0.05% trypsin and 0.01% EDTA.

Annexin V/7-AAD Staining

HeLa cells were cultured at confluence into 25 cm^2 flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) in a complete medium. Then, the medium was replaced by fresh complete medium containing EGCG (50 mcg/ml), FA (900 nM), B12 (1,000 nM), HA (10 mcg/ml) or EGCG (50 mcg/ml) + FA (900 nM) + B12 (1,000 nM) + HA (10 mcg/ml). After 48 hours of treatment, the cells status was assessed by the FITC Annexin V Apoptosis Detection Kit I according to the instructions of the manufacturer (BD Pharmingen™).

Expression of p53, E6/E7 and Rb Messenger RNA

HeLa cells were cultured at confluence into 25 cm^2 flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) in a complete medium. Then, after 24 hours, the medium was replaced by fresh complete medium containing EGCG (50 mcg/ml), FA (900 nM), B12 (1,000 nM), HA (10 mcg/ml) or EGCG (50 mcg/ml) + FA (900 nM) + B12 (1,000 nM) + HA (10 mcg/ml). After 48 hours of treatment, the total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. 1 µg of total RNA was reverse transcribed into complementary (cDNA) using FastGene Scriptase II cDNA kit (Nippon Genetics Europe, Germany). 1 µg of total cDNA was used for qPCR using the iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA, USA). mRNA levels were standardized using beta-actin cDNA. The ratio was compared between treated and control conditions and the analysis was performed in triplicate for each sample. The results were expressed as fold change with respect to control values.

The primers used for qRT-PCR of Rb and E6/E7 mRNA were previously published[15], as well as the primers for β-actin[16].

Statistical Analysis

GraphPad Prism 8 (GraphPad, La Jolla, CA, USA) was used for statistical analyses and drawing graphs. One-way ANOVA was computed, and Bonferroni post-test was used to evaluate any significant (p<0.05) difference reported in this paper. All the assays were repeated at least three times; all histograms show the mean value ± sd. When the differences between experimental conditions are shown as the "number of folds", sd was consequently transformed.

Results

The Association of EGCG, FA, B12 and HA Induces Apoptosis in HeLa Cells

We investigated the induction of apoptosis by the association of EGCG, FA, B12 and HA through the analysis of Annexin V. The 48-hour treatment with the MIX (****p<0.0001) and HA (*p<0.05) determined an increased apoptosis compared to control, EGCG, and FA conditions alone. Moreover, we found that MIX induced an additive statistically significant difference on the induction of apoptosis with respect to EGCG alone ($^{\#\#\#\#}p$<0.0001) (Figure 1).

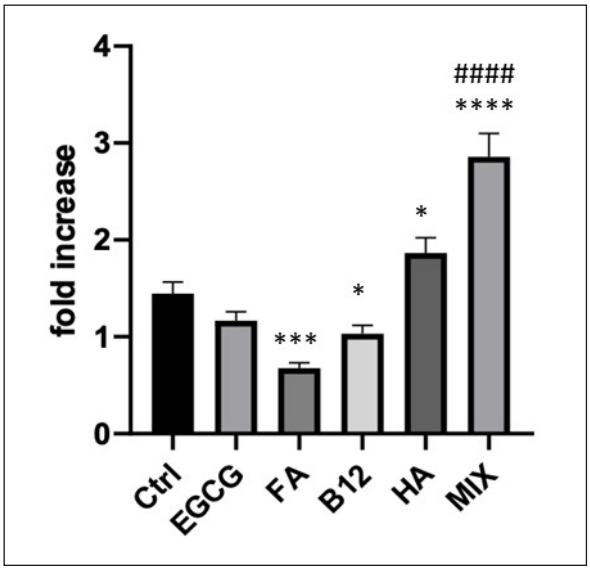

Figure 1. The effect of EGCG, FA, B12, HA and their combination (MIX) on apoptosis in HeLa cells after 48 hours of treatment. Apoptotic rate was determined with FITC Annexin V Apoptosis Detection Kit I. Histograms show the percentage of apoptotic cells (Annexin V+/7-AAD); each column representing the mean value ± SD of three independent experiments (*p<0.05 *vs.* ctrl; ****p<0.0001 *vs.* ctrl; $^{\#\#\#\#}p$<0.0001 *vs.* EGCG).

The Association of EGCG, FA, B12 and HA Modulates Gene Expression Levels of p53, Rb and E6/E7

We investigated the effect of the association of EGCG, FA, B12, and HA on gene expression levels of p53, Rb, and E6/E7 in HeLa cells (Figure 2). The expression levels of p53 significantly increased with B12 compared to control (*p<0.05) but, more interestingly, it strongly increased with the association of EGCG, FA, B12, and HA (***p<0.001) compared to control conditions (Figure 2A).

All the tested molecules except for HA, and their combination significantly reduced the expression levels of E6/E7 gene compared with control conditions (Figure 2B). Finally, neither the single molecules nor the combined treatment influenced gene expression levels of Rb after a 48-hour treatment (Figure 2C).

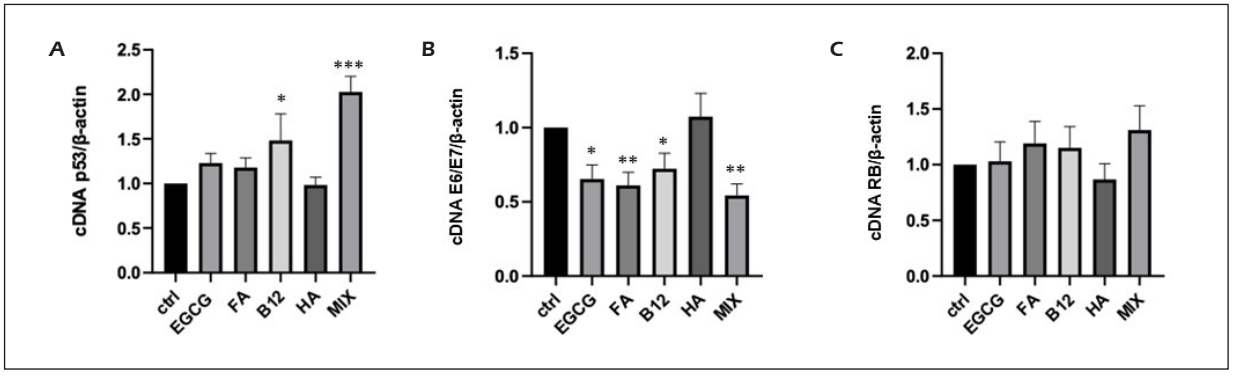

Figure 2. Real-time PCR for p53 (**A**), E6/E7 (**B**) and Rb gene (**C**) in Hela cells treated with EGCG (50 mcg/ml), FA (900 nM), B12 (1,000 nM), HA (10 mcg/ml) or EGCG (50 mcg/ml) + FA (900 nM) + B12 (1,000 nM) + HA (10 mcg/ml). (MIX). Quantitative RT-PCR sample values were normalized for the expression of β-actin and reported as fold change *vs.* Ctrl set as 1. The results are the mean ± sd N=3; *p<0.05; **p<0.01; ***p < 0.001 *vs.* Ctrl.

Discussion

We found out for the first time that the association of EGCG, FA, vitamin B12, and HA significantly increases apoptosis in HeLa cells, by upregulating p53 and downregulating E6/E7 expression, respectively.

After breast cancer, cervical cancer is the second most common female cancer worldwide. Various strains of the HPV, mainly HPV16 and 18, play a crucial role in the pathogenesis of most cervical cancers[17]. Cervical cancer is characterized by a well-defined pre-malignant phase, which represents a spectrum of histological abnormalities ranging from CIN1(mild *dysplasia*) to CIN2 (moderate dysplasia) and CIN3 (severe dysplasia/carcinoma in situ). At this last stage, integration of the HPV genome is established with a consequence loss of negative-feedback control of oncogene expression, following disruption of the viral regulatory early gene *E2*. Although the prevalence of integrated forms varies with the infecting HPV type, HPV18 integration seems virtually complete in women with cervical intraepithelial neoplasia grade 3 (CIN3) or invasive disease[12]. Despite prevention programs, an effective therapy to counteract HPV infection and consequences of its persistence does not exist. In this regard, some scholars[18] found that some natural molecules, such as EGCG, have pro-apoptotic effects on tumor cells, and for this reason, may represent a valid aid. Recently, green tea has emerged as a potential chemo-preventive agent for several types of cancers, including HPV-related ones. EGCG is the most abundant polyphenol of green tea and previous evidence[19] demonstrated its beneficial properties. Topical pharmaceutical preparation containing EGCG, have already demonstrated their positive efficacy for the treatment of external genital warts (EGW) and perianal warts. Several studies[20,21] have indeed demonstrated that EGCG induces a statistically significant clearance of all baseline EGW and anogenital warts when compared to placebo. Moreover, oral EGCG is potentially protective for patients with HPV-infected cervical lesions, or cervical intraepithelial neoplasia, thus reducing the risk of cervical cancer[22].

Prior studies[23,24] evaluated the association between diet and the risk of HPV infection and its persistence. Chi et al[23] for example, reported that a higher intake of nutrients with antioxidant and antiviral functions may prevent the progression of HPV infection to high-grade cervical intraepithelial neoplasia. Nutrients, such as folate and vitamin B12, may have a role in regulating viral integration and gene stability due to their involvement in DNA synthesis, repair, and methylation[24]. Moreover, a diet rich of folic acid and B12 seems to have a protective effect against the HPV transmission. HA has a well-known reparative action: for this reason, it can play a crucial protective role as a physical barrier blocking HPV entry by restoring the integrity of the damaged epithelium or mucosae. Moreover, to date, the application of vaginal HA represents one of the most used approaches to restore the re-epithelization of the cervix, thus helping spontaneous viral clearance, mainly in conditions of LSILs. However, in the case of persistent infections, HA may represent a valid approach in adjuvating the efficacy of other compounds[25] and reducing the number of lesions. By combining the oral supplementation with the administration of the HA soft gel caps, the clearance of the virus increases and the persistence of LSIL/CIN1 lesions reduces during a long-term follow-up[25].

Nonetheless, a specific therapy to counteract HPV infection and consequences of its persistence does not still exist; moreover, the proposed natural molecules, have never been tested in the association.

The authors are aware that the study presents some limitations, mainly related to the use of a single cell line, the HeLa cells. However, a human cervical carcinoma cell line, represent a well-established and widely used model of persistent infection. Further studies in other cellular and then animal models are of course advisable, but the model used here is sufficient as a first screening to test our original hypothesis.

Therefore, for the first time, in this study, we investigated the combination of EGCG + FA + B12 + HA in HeLa HPV-positive cells. HeLa cells represent a model of persistent HPV infection because they contain multiple copies of integrated HPV18 DNA. Results revealed that this association significantly increased apoptosis ($p<0.05$), much more than every single molecule and EGCG alone. Several genes and proteins are involved in the regulation of the apoptosis process, but the master regulators are p53 and Rb. P53 specifically, is considered the "guardian of the genome", since it preserves cells from cellular damage and regulates the expression of genes involved in processes of DNA repair, cell division and cell death[25]. When HPV persists and the virus integrates its DNA in the host genome, the

viral oncoproteins (E6/E7) abrogate the activity of p53 and Rb. Therefore, restoring their activity is essential for a chemopreventive effect.

Conclusions

Our results evidence for the first time that the association of EGCG, FA, B12, and HA, significantly upregulates apoptosis *via* p53 expression ($p<0.001$) and downmodulates the viral genes E6/E7 in HeLa HPV-positive cells, meanwhile they have no effect on Rb expression. Although further studies are necessary to deepen the effects of such natural molecules, the possibility to induce apoptosis of tumoral cells and the functional modulation of both p53 and viral genes E6/E7 represents a promising strategy to counteract HPV infection and consequences of its persistence.

Conflict of Interest

Vittorio Unfer, Sara Proietti and Elisa Lepore are employees at Lo.Li. Pharma srl.

Ethics Approval

Not applicable. The study has been conducted following the Helsinki declaration and its later amendments.

Informed Consent

Not applicable.

Authors' Contribution

Conceptualization, A.F. and A.F.; data curation, G.C, V.U, S.P. and E.L.; formal analysis, S.P, E.L., A.F.; investigation and methodology G.C., V.U.; project administration, V.U., A.F., A.F.; writing-original draft, A.F.; writing and editing, S.P., E.L., A.F. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data presented in this study are available on request from the corresponding author.

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