Original Article

High-risk human papillomavirus infections in colorectal cancer in the Syrian population and their association with Fascin, Id-1 and P-cadherin expressions: A tissue microarray study

Lina Ghabreau[1,2], Eric D. Segal[3], Amber Yasmeen[3], Amal Kassab[3], Nizar Akil[1,2], Ala-Eddin Al Moustafa[2,3,4,5]

[1]Department of Pathology, Faculty of Medicine/University of Aleppo, Aleppo, Syria, [2]Syrian Research Cancer Centre of the Syrian Society against Cancer, Aleppo, Syria, [3]Segal Cancer Centre of the Lady Davis Institute/JGH/McGill University, [4]Department of Oncology, McGill University, Montreal, Quebec, Canada and [5]Department of Mechanical Engineering, Concordia University, Montréal, Quebec, Canada

ABSTRACT

Background: High-risk human papillomaviruses (HPVs) can be regarded as important risk factors for colorectal carcinogenesis and metastasis. Alternatively, earlier studies have reported that Fascin, Id-1 and P-cadherin genes are important regulators of cell invasion and metastasis in several human carcinomas, including colorectal. In order to investigate the correlation between the presence of high-risk HPVs and Fascin, Id-1, and P-cadherin genes in human colorectal cancer (CRC) in the Syrian population, we examined the incidence of high-risk HPV types (16, 18, 31, 33 and 35) and their association with Fascin, Id-1 and P-cadherin expression. **Materials and Methods:** A total of 78 blocks from CRC Syrian patients were used in this study. These blocks were analyzed using polymerase chain reaction (PCR) and tissue microarray (TMA) analyses for the presence of high-risk HPVs and Fascin, Id-1 as well as P-cadherin expression, respectively. **Results:** We found that high-risk HPVs were present in 42 samples (53.84%), which represent the majority of invasive CRC cases; the most frequent high-risk HPV types in the Syrian population are 16, 33, 18, 35 and 31 respectively. Furthermore, the expression of E6 onco-protein of high-risk HPVs was found to be correlated with Fascin, Id-1 and P-cadherin expression/over-expression in the majority of CRC tissue samples. **Conclusion:** These data reveal that high-risk HPVs are present in human CRCs in the Syrian population, and their presence is associated with invasive and metastatic phenotype.

Keywords: Colorectal cancer, high-risk HPV, fascin, Id-1 and P-cadherin

INTRODUCTION

Colorectal cancer (CRC) is the third most common type of cancer, with approximately 1.2 million new cases diagnosed annually worldwide, accounting for about 9.7% of all cancer cases (WHO). Evidence from recent studies suggests that environmental conditions and lifestyle, in addition to sequential genetic changes and possibly viral components, are major risk factors for colorectal cancer. Human papillomaviruses (HPVs) have been established as etiological agents of invasive cervical cancer, as roughly 96% of these cancers are positive for high-risk HPVs, which is the most common viral sexually transmitted infection worldwide.[1,2] Persistent infection with high-risk HPVs is necessary for the development of premalignant lesions and/or progression of the disease.[3] Furthermore, high-risk HPVs have carcinogenic effects at several other anatomical sites in women and men such as head and neck as well as CRC,[4-6] studies showed that high-risk HPVs are present

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Address for correspondence: Prof. A-E. Al Moustafa, Department of Mechanical and Industrial Engineering, Concordia University, 1455 Maisonneuve Blvd., West, Montreal, QC, H3G 1M8, Canada. E-mail: ala-eddin.almoustafa@mcgill.ca, alaeddin.almoustafa@gmail.com

in roughly 30% and 91% of these cancers, respectively.[5-7] The high-risk HPV E6 and E7 onco-proteins, which are consistently expressed in infected cells, bind and inactivate the p53 and pRb tumor suppressors, respectively,[8] as well as other proteins leading to cell cycle deregulation.[9] This results in genomic instability and has been implicated in cancer development and progression.

The CRC, like other human carcinomas, is characterized by a marked propensity for distant metastases, which is a major cause of death in CRC patients. The formation of metastases is a multistep process, in which malignant cells disseminate from the primary tumor to colonize distant organs. This is a highly inefficient and complex process, which involves early steps of tumor cell invasion of the microenvironment, entering the bloodstream, survival during migration, and extravasation into distant organs.[10] Therefore, each step in metastasis requires specific genetic and epigenetic changes. For instance, several studies reported that Fascin, Id-1 and P-cadherin genes are important regulators in the progression of several human carcinomas including CRC.[11-17] Alternatively, we recently demonstrated that Id-1 is an important target for E6/E7 onco-proteins of high-risk HPVs in human breast and cervical metastatic cancer; moreover, we reported that E6/E7 of high-risk HPV type 16 convert non-invasive/non-metastatic cancer cells into invasive and metastatic ones *in vitro* and *in vivo*, respectively.[18-20] Likewise and using cDNA microarray analysis, we found that Fascin, Id-1 and P-cadherin are up-regulated in human E6/E7-infected cancer cells compared to their wild-type cells [unpublished data]. These studies suggest that Fascin, Id-1 and P-cadherin promote the development and the progression of human HPV-infected cancer cells.

This study aims to recognize the specific types of high-risk HPV infections present in Syrian CRC patients and their association with Fascin, Id-1 and P-cadherin expression.

MATERIALS AND METHODS

HPV detection and type specification

A total of 78 blocks from CRC patients (41 females and 36 males) with a median age of 49 years were used in this study. Formalin fixed (buffered neutral aqueous 10% solution), paraffin-embedded tumor materials were obtained from the Department of Pathology/University of Aleppo and its hospitals. The use of these specimens and data in research was approved by the Ethics Committee of the Faculty of Medicine of Aleppo University. Five μg of purified DNA (from each block) was analyzed for HPV by multiplex PCR targets to the conserved L1 region of the viral genome by use of PGMY09/11 L1 primer pools.[19] In parallel, we used specific primers for E6 and E7 genes to detect HPV types 16, 18, 31, 33 and 35, while, specific primers for the GAPDH gene were used as an internal control.[20,21] PCR products were denatured in 0.13N NaOH and hybridized to an immobilized HPV probe array using an extended reverse line-blot assay for HPV genotyping (Roche Molecular Systems, Inc., Alameda, California, USA) of five HPV types classified as high-risk HPVs (types 16, 18, 31, 33 and 35) as described by Begum *et al*.[19]

Tissue microarray

The tissue microarray (TMA) construction was performed as described by Akil *et al*.[20] Briefly, tissue cylinders with a diameter of 0.6 mm were punched from representative tumor areas of the tissue block using a semiautomatic robotic precision instrument. Two sections of the TMA blocks were transferred to an adhesive-coated slide system (Instrumedics Inc., Hackensack, New Jersey, USA). Slides of the finished blocks were used for immunohistochemistry analysis.

Immunohistochemistry

Immunohistochemical procedures examining the expression of Fascin, Id-1, P-cadherin and E6 were carried out using standard procedures as previously described by our group.[22] Primary specific antibodies were obtained from Santa Cruz Biotechnology and Calbiochem (Santa Cruz and San Diego, California, USA). Briefly, TMA sections were deparaffinized and rehydrated, and endogenous peroxidase activity within the rehydrated tissue was blocked with a solution of 3% hydrogen peroxide in methanol for 10 min at room temperature. The TMA slides were cooled and equilibrated in Optimax™ wash buffer, then incubated overnight at 4°C with primary antibodies for Fascin, Id-1, P-cadherin and E6. Sections were then washed, and the appropriate secondary HRP-conjugated antibody was applied for 1 h at room temperature (Calbiochem, San Diego, California, USA). The slides were counterstained with hematoxylin and mounted.

RESULTS

In order to determine the role of high-risk HPV infections in human CRC in the Middle East, we investigated the presence of high-risk HPV types 16, 18, 31, 33 and 35 in a cohort of 78 CRC samples from the Syrian population by PCR analysis using PGMY09/11 L1 primer pools and specific primers for their E6 and/or E7 genes, as previously described by our group.[20,21] Our study revealed that 42 (53.84%) of the 78 samples are HPV positive and 29 (37.17%) of these specimens were co-infected with more than one HPV type [Table 1]. We found that HPV types 16, 18 and 31 were present in 36, 30 and 6 cancer tissues, respectively [Table 2]. In parallel, 36 and 15 cancer tissues were positive for HPV types 33 and 35, respectively [Table 2].

Next, we examined the expression of the E6 onco-protein of high-risk HPVs along with Fascin, Id-1 and P-cadherin expressions, which are important regulators of cell invasion and metastasis,[11,14,16] in all our CRC tissue samples by immunohistochemistry using tissue microarray methodology, in order to assess the correlation between these genes. We found that E6 expression is correlated with Fascin, Id-1 and P-cadherin, expression/over-expression in 92.85%, 85.71% and 78.57% of invasive CRC tissues, respectively, [Table 3 and Figure 1]. Although we presume that the few cases of *in-situ* CRC, which are HPV-positive, may ultimately progress into invasive carcinomas. Moreover, to confirm the association between E6/E7 of HPV types 16, 18, 31, 33 and 35 and Fascin, Id-1 and P-cadherin, we investigated the presence of E6 and/or E7 of these viruses by PCR, using specific primers for E6/E7 genes, as described in the materials and methods section. By means of this analysis, we were able to validate our data regarding the presence of E6/E7 of HPV types 16, 18, 31, 33 and 35 and their association with Fascin, Id-1 and P-cadherin over-expression in the majority of invasive CRC tissues [Table 3].

Table 1: High-risk HPV type 16, 18, 31, 33 and 35 detection in CRC by PCR

	Tested cases	Positive	Percentage
Colorectal cancer tissues	78	42	53.84

The incidences of these viruses were found in 42 samples out of 78 examined using PGMY09/11 L1 primer pools and specific primers for E6 and/or E7 of each HPV type (P<0.0001)

Table 2: Presence of HPV type 16, 18, 31, 33 and 35 in CRC

CRC number of cases	High-risk HPV types				
	16	18	31	33	35
78	36/78	30/78	6/78	36/78	15/78

High-risk HPVs (16, 18, 31, 33 and 35) are present in the majority of invasive CRC tissues (P<0.001, P<0.001, P<0.001, P<0.001 and P<0.001, respectively). Furthermore, it is clear that HPV type 16 and 33 are the most common in CRC in the Syrian population

Table 3: Correlation between the expression of the E6 onco-protein of high-risk HPVs and Fascin, Id-1 and P-cadherin in CRC tissues using tissue microarray analysis

Number of CRC studied	Number of positive samples			
	E6	Fascin	Id-1	P-cadherin
78	42/78	42/78	36/78	36/78

The expression of the E6 onco-protein is associated with Fascin, Id-1 and P-cadherin expression/over-expression in 92.85%, 85.71% and 78.57% of CRC cases, respectively, (P<0.001,P<0.001, P<0.001). We noted that there is no association between E6 and Fascin, Id-1 and P-cadherin in only one, two and three cases, respectively

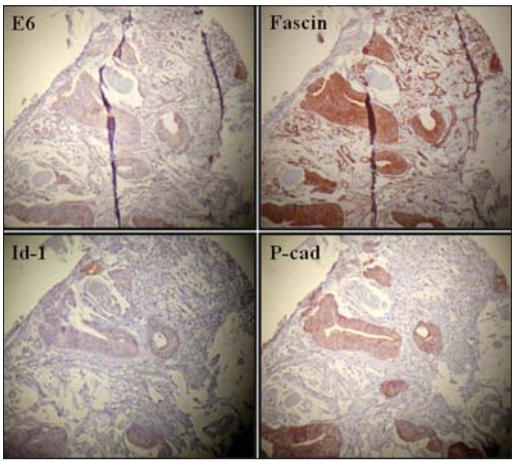

Figure 1: Association between the presence of high-risk human papilloma virus (HPV) type 16 and Fascin, Id-1 and P-cadherin expression/over-expression in human CRC in a sample patient. We noted that E6 expression of HPV type 16 is correlated with Fascin, Id-1 and P-cadherin expression/over-expression in this case of CRC tissue using tissue microarray methodology. Magnification is 200×. The presence of HPV type 16 was confirmed by PCR using specific primers for the E6 gene of this virus

DISCUSSION

This is, to the best of our knowledge, the first study on the presence of high-risk HPVs and their association with Fascin, Id-1 and P-cadherin expression in CRC in the Arab world. It has been shown that the presence of certain types of HPVs is related to specific geographic locations;[20] for example, previous studies on CRC have reported that high-risk HPV types 16 and 18 are the most frequent in the United States, Argentina and Italy;[23-25] whereas, HPV type 16 and 33 are present in the majority of French population, as a single infection.[7] In parallel, investigation on the presence of high-risk HPV in Turkey revealed that HPV types 18 and 33 are the most frequent types in CRC patients.[26] On the other hand, the presence of high-risk HPVs in human CRC tissues varies from 44% to 91%.[24,7] However, and, based on few investigations, the significance of such findings remains controversial.[27] In this study, we report that high-risk HPVs are present in 53.84% of CRC in the Syrian population; moreover, HPV types 16 and 33 are the predominant viruses of the high-risk HPV family members studied. Therefore, our data confirm that specific types of high-risk HPV infection, in CRC tissues, are related to specific geographic locations. Nevertheless, we believe that future studies on a larger number of samples will be necessary to confirm the incidence of HPVs in CRC patients of the Arab populations since our study is the first in this region.

Regarding the association between high-risk HPVs and tumor aggressiveness, recent studies, including ours, have reported that the presence of HPV type 16, in human cervical,[1,21] breast[20,28] and head and neck[5] cancer, is correlated with invasive carcinomas. On the other hand, it has been demonstrated that Fascin, Id-1 and P-cadherin play a significant role in the regulation of cell invasion and metastasis of several human carcinomas including CRC.[11-17] In parallel, we reported that E6/E7 onco-proteins of HPV

type 16 convert non-invasive breast and cervical cancer cells into an invasive phenotype;[18,29] this is accompanied by an over-expression of Fascin, Id-1 and P-cadherin. In this study, we investigated the association between high-risk HPVs and Fascin, Id-1 and P-cadherin expression in CRC tissues from the Syrian population. We report, for the first time, that the presence of E6/E7 onco-proteins of HPV types 16, 18, 31, 33 and 35 are associated with Fascin, Id-1 and P-cadherin expression/over-expression in cancer tissues in comparison with adjacent normal tissues. Previous investigations revealed that Fascin, Id-1 and P-cadherin are expressed/over-expressed in CRC, but not in normal CR tissues.[13,15,16] Separately, we revealed that E6/E7 of HPV type 16 affects Id-1 deregulation via the activation of its promoter.[18] We believe that E6/E7 of high-risk HPVs deregulate several genes including Fascin and P-cadherin through the activation of their promoters. Accordingly, the present study clearly suggests that Fascin, Id-1 and P-cadherin are downstream targets of E6/E7 of HPV types 18, 31, 33 and 35 in human CRC progression.

In conclusion, we demonstrate that high-risk HPV types 16, 18, 31, 33 and 35 are present in human CRC in the Syrian population. In addition, this study, combined with our previous studies on the Syrian population, reveals that HPV types 16 and 33 are the most dominant types of HPV infection. In parallel, we report that Fascin, Id-1 and P-cadherin are important targets for E6/E7 onco-proteins of HPV type 16, 18, 31, 33 and 35 in CRC cancer cells. Our findings provide a new basis for understanding the mechanisms of high-risk HPV infections and their relation to human CRC development and progression. However, we firmly believe that further studies are required to elucidate the presence and pathogenesis role of high-risk HPV in human CRC cancer, especially since HPV vaccines for only two high-risk HPV types (16 and 18) are available at present. Therefore, we strongly advocate that a future implementation of routine HPV vaccination, using the actual or new generation of HPV vaccines in Syria,[30] can significantly reduce the burden of HPV-associated diseases as well as the national healthcare expenditures.

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