RESEARCH COMMUNICATION

Global DNA Methylation in Precancerous and Cancerous Lesions of the Uterine Cervix

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Abstract

Introduction: Persistent human papillomavirus (HPV) infection is the primary causal agent in the development of the uterine cervix carcinoma. Nevertheless, only a minority of high-risk HPV-associated lesions progress to cervical cancer, suggesting involvement of other molecular alterations. Among putative changes, aberrant methylation might be a crucial event. Design: Paraffin-embedded samples of benign lesions, cervical intraepithelial neoplasia (CIN) and invasive squamous cell carcinomas (SCC) were analyzed for DNA 5-methylcytosine content by immunohistochemistry with anti-5-methylcytosine antibodies and by high-performance liquid capillary electrophoresis (HPCE). Results: No significant difference of DNA 5-methylcytosine content was observed between normal tissues, benign lesions, low-grade lesions and high-grade lesions (p=0.6). In contrast, DNAs extracted from invasive SCC were hypomethylated when compared with normal and preneoplastic lesions (p=0.0004). An association between global DNA hypomethylation and the SCC stage was confirmed by HPCE. Conclusions: The transition from CIN lesions to invasive carcinoma seems to be closely linked to global DNA hypomethylation, which could be a useful marker of invasive uterine cervical lesions.

Keywords: Uterine cervix - CIN - cancer - DNA hypomethylation

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Introduction

Cervical cancer is an important cause of death in women worldwide and is the main cancer among women in most developing countries (zur Hausen, 2002; Parkin, 2006). The persistent human papillomavirus (HPV) infection is the primary causal agent in the development of uterine cervical carcinoma and its precursor lesions (zur Hausen, 2002). The viral oncogenes (E6, E7) of high-risk HPV have the ability to bind to cellular regulatory proteins, especially the tumor suppressor gene product p53 and the hypophosphorylated retinoblastoma protein (pRb) (zur Hausen, 2000; Dell, 2001; zur Hausen, 2001). Several studies demonstrated the usefulness of the p16^{ink4a} overexpression is an indicator of an aberrant expression of viral oncogenes and may serve as a marker for uterine cervix early diagnosis (Klaes et al., 2001; Wentzensen et al., 2005; Missaoui et al., 2006; Cuschieri and Wentzensen, 2008; Missaoui et al., 2010). Nevertheless, only a minority of high-risk HPV-associated lesions progress to uterine cervix carcinoma (Ostor, 1993). Thus, HPV infection alone is probably insufficient for complete neoplastic transformation of uterine cervical cells, suggesting the involvement of other molecular alterations. Among the putative molecular alterations involved in this neoplastic process, aberrant methylation might be a crucial event (Tost, 2009). Tumorigenesis is very often associated with a global reduction of 5-methylcytosine content (Gama-Sosaet al., 1983). Global genomic hypomethylation has been found in many types of human cancer, including prostate metastatic tumors (Bedford and van Helden, 1987), leukocytes from B-cell chronic lymphocytic leukemia (Wahlfors et al., 1992) and hepatocellular carcinomas (Lin et al., 2001).

In this study, we analyzed the global DNA methylation level in uterine cervix lesions in order to investigate whether this epigenetic event is involved in the process of cervical carcinogenesis and whether it might be used as marker of cervical lesions.

Materials and Methods

Tissue samples

Seventy specimens were retrieved from the surgical pathology files of Pathology Department, Farhet Hached University Hospital, Sousse, Tunisia, and the Pathology Department, Edouard Herriot University Hospital, Lyon, France.

The cases studied were distributed into the following groups, according to the World Health Organization

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(WHO) Classification of Tumors of the Breast and Female Genital Organs, 2003 (Tavassoli and Devilee, 2003): normal uterine cervix (n=10), benign lesions including squamous metaplasia, chronic cervicitis and atypia of repair (n=20), cervical intraepithelial neoplasia 1 (CIN1) (n=6), CIN2-3 (n=19) and invasive squamous cell carcinomas (SCC) (n=15). The cases were selected randomly, and the slides were reviewed by two pathologists (Dr. Lucien Frappart and Dr. Bernard Fontanière).

The material consisted of uterine cervix biopsy specimens obtained from women undergoing evaluation for abnormal smears and constituted of representative preneoplastic or neoplastic cells in each case. The ten normal uterine cervix cases were selected mainly among women in their reproductive years who scheduled for hysterectomy due to benign gynecological abnormalities. The study protocol was reviewed and approved by the institutional scientific and ethical review committees.

All tissues had been routinely fixed in 4% buffered formalin and paraffin embedded. In the current study, two control hematoxylin and eosin-stained slides were realized before and after tissue section to control the uterine cervix lesion presence in material used for DNA extraction. Control slides were reviewed by two pathologists.

Global DNA methylation immunodetection

The immunostaining procedure was carried out as already described for other issues (Ghabreau et al., 2004; Perrin et al., 2007). Normal tissues, benign lesions, neoplastic and preneoplastic uterine cervix lesions were fixed in formalin, paraffin embedded. One or two paraffin blocks containing representative portions of the tumors were selected for each case, and 4-μm-thick sections were obtained. Sections were incubated with antibodies against anti 5-methylcytosine (generous gift of Dr A. Niveleau). The remaining part of the procedure was performed as already previously described (Ghabreau et al., 2004; Perrin et al., 2007). Three independent investigators (Dr. Lucien Frappart, Dr. Bernard Fontanière, and Dr. Nabiha Missaoui) carried out a semiquantification of the immunostaining with both the staining intensity (0, no staining; 1, weak staining intensity; 2, intermediate; and 3, strong staining intensity) and the percentage of positively stained tumor cells (0, no positive cells; 1, <25%; 2, 26-50%; 3, 51-75%; 4, 76-99%; 5, 100% positive tumor cells). After multiplication of both values, the results were graded from 0 (no reactivity in tumor cells) to 15 (100% positive tumor cells with strong staining intensity) (Missaoui et al., 2006; Missaoui et al., 2010).

DNA extraction

Sections were obtained from formaldehyde fixed, paraffin-embedded uterine cervix samples (5 to 10 μm thick, per block). Although our material consisted of biopsies constituted of representative preneoplastic or neoplastic cells in each case; when necessary, a macrodissection of tissue areas was performed to avoid stromal contamination, mainly in normal tissues and benign lesions. DNA from both tumor and non-neoplastic tissues was extracted using a commercial kit (QIAamp® DNA Kit, QIAGEN, Courtaboeuf, France) as previously described (Perrin et al., 2007).

Global 5-methylcytosine quantification

The 5-methylcytosine (mC) content was determined by high-performance capillary electrophoresis (HPCE) as previously described (Perrin et al., 2007; Fraga et al., 2002). In brief, precipitated DNA samples were enzymatically hydrolyzed in a final volume of 5 μl. Samples were then directly injected in a Beckman MDQ high-performance capillary electrophoresis apparatus and the mC content was determined as the percentage of mC in the total cytosine: mC peak area x 100 / (C peak area + mC peak area).

Statistical analysis

The data were examined by Chi-square statistics using the Epi Info 2002 software as previously described (Missaoui et al., 2006; Missaoui et al., 2010). Probability values of 0.05 or less were considered statistically significant.

Results

Global DNA methylation immunodetection

Immunolabeling was observed in basal and parabasal cells in normal squamous epithelium, benign lesions as well as in all CIN1 (mean score: 11.6 ± 0.7 and 11.7 ± 0.6, respectively) (Figure 1). In CIN2-3 samples, the entire epithelium thickness was stained (mean score: 11.9 ± 0.4). No statistically significant difference was observed between normal epithelium, benign lesions, CIN1 as well as CIN2-3 (p=0.6). However, immunostaining intensity was reduced in invasive SCC (mean score 7.2 ± 3.3) when compared with normal and benign uterine cervix tissues (p=0.0004), CIN1 lesions (p=0.0004) and CIN2-3 lesions (p=0.0002) (Figure 1). In invasive SCC, undifferentiated cells exhibited a lower immunolabeling with anti-5-methylcytosine antibodies.

Global 5-methylcytosine quantification

As HPCE technique requires a high DNA quantity extract for the 5-methylcytosine content analysis (1μg/μl), only sufficient DNA quantity extract were retuned. Thus, HPCE was determined only for 19 uterine cervix specimens including 4 normal tissues, 10 precancerous lesions (5 CIN1 and 5 CIN2-3) and 5 invasive SCC. The

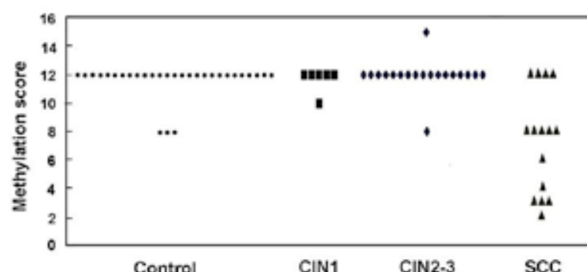

Figure 1. Global DNA Methylation in Uterine Cervix Lesions. Control, normal squamous epithelium and benign lesions; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma. Y axis correspond to the immunolabeling score obtained for DNA methylation

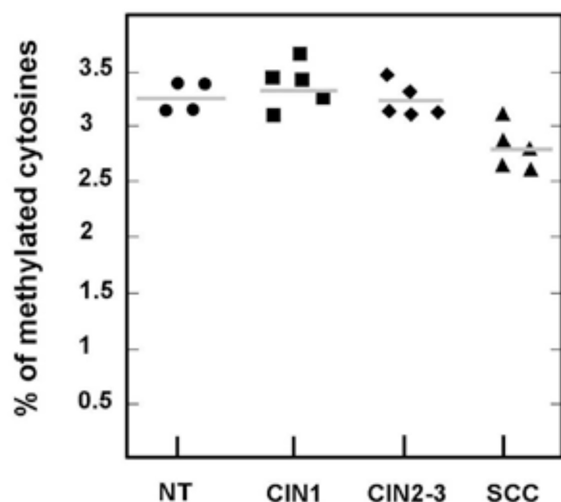

Figure 2. 5-Methylcytosine Content Assessed by High-performance Capillary Electrophoresis (HPCE) in Uterine Cervix Lesions. Mean value for each tissue type was represented by horizontal bar. NT, normal tissue; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma

5-methylcytosine immunolabeling score was 12 for cases of normal tissue, CIN1 and CIN2-3. Invasive SCC were scored 12 (one case), 8 (one case), 4 (one case) and 3 (2 cases).

Using HPCE analysis, no significant difference of DNA methylation was observed between normal tissue, low- and high-grade uterine cervix lesions with 3.26%, 3.37% and 3.22%, respectively (p>0.05) (Figure 2). In contrast, DNAs extracted from invasive SCC were hypomethylated when compared with normal and precancerous uterine cervix tissues; the percentage of methylated cytosines falling down to 2.81%, (p=0.0004) .

Discussion

Global DNA methylation level analyzed using an antibody directed against 5-methylcytosine showed no statistically significant immunoreactivity difference between normal epithelium, benign lesions, CIN1 as well as CIN2-3. However, immunostaining intensity was significantly reduced in invasive SCC (mean score 7.2 ± 3.3) when compared with normal and benign uterine cervix tissues (p=0.0004), CIN1 lesions (p=0.0004) and CIN2-3 lesions (p=0.0002). These findings suggest that global DNA hypomethylation occurs only in invasive uterine cervix carcinoma. Thus, global DNA hypomethylation would be a useful marker of invasive uterine cervix carcinogenesis.

In order to confirm the potential interest of this marker, we investigated global DNA methylation level using HPCE. Base composition analysis showed global DNA hypomethylation in invasive SCC when compared with normal and precancerous uterine tissues. The percentage of methylated cytosines falling down to 2.81%, (p=0.0004). Thus, both methods base composition analysis and immunohistochemistry, lead to the same conclusion: the loss of 5-methyl-deoxycytidine is a characteristic of invasive SCC of the uterine cervix.

Previously, DNA methylation was evaluated by the level of radio-labeled methyl group incorporation driven by SssI DNA methyltransferase in a study published by Kim et al., (1994). This method is very sensitive, the authors observed a 7 fold increase of incorporation of methyl groups between normal and cancer. Although these data clearly indicated that uterine cervix cancers were hypomethylated, the absolute values of methyl loss cannot be deduced from this method.

Many studies have indicated that cancer cells loose between 10 and 30% of their methyl groups (Ehrlich, 2002). These percentages indicate that the incorporation of methyl groups is not proportional to the number of methyl groups on DNA molecules. Moreover, Kim et al. study (Kim et al., 1994) and as our own data indicate that the major difference was observed between cancer samples and the other samples. Using computer-assisted analysis of the immunostaining (de Capoa et al., 2003), many cell types were analyzed including cultured cells, human peripheral blood B-lymphocytes from normal and chronic lymphoid leukemia (CLL) samples and uterine cervix biopsies. Data obtained from these series suggested that demethylation was associated with tumor progression (de Capoa et al., 2003). However, in this study a very limited number of uterine cervix samples were analyzed and the method used for DNA methylation quantification was different from the method used in our own manuscript. Therefore, a direct comparison between the two sets of data is very difficult.

More recently, using the semiquantification method of the immunostaining (both the staining intensity and the percentage of positively stained tumor cells), Piyathilake et al. (Piyathilake et al., 2006) demonstrated that mandatory fortification with folic acid in the United States has not resulted in a change in the degree or the pattern of global DNA methylation in cells involved in cervical carcinogenesis.

The etiology of uterine cervix cancers is mainly associated with infection by the high-risk HPV 16 and 18. E7 is the major transforming protein of HPV and it has been shown that this protein associate in vitro and *in vivo* the DNA-Methyl-Transferase 1 (DNMT1) (Burgers et al., 2007). DNMT1 is the main enzyme responsible of the methylation of newly replicated DNA and thus directly involved in the maintenance of DNA methylation patterns during cell growth (Goyal et al., 2006). The in vitro methyltranferase activity of DNMT1 is stimulated by the E7 oncoprotein (Burgers et al., 2007), suggesting that HPV infection may interfere with the DNA methylation machinery.

DNA hypomethylation has been observed in most of the cancer tissues analyzed (Ehrlich, 2002). It has been suggested that local CpG islands hypermethylation at specific loci occurs independently of global DNA hypomethylation (Ehrlich et al., 2002; Ehrlich et al., 2002; Widschwendter et al., 2004); nevertheless loss of 5-methyl-deoxycytidine is an early event in carcinogenesis (Ehrlich, 2002). Prostate cancers seem to represent an exception to this rule, since coordinate hypermethylation of *GSTP1, RARB2, RASSF1A*, and *APC* CpG islands precedes the hypomethylation of the repeated elements (Florl et al., 2004).

100.0
75.0
50.0
25.0
0

In summary, global DNA hypomethylation would be a useful marker of invasive uterine cervix carcinogenesis. Our findings suggest that the transition from uterine CIN lesions to invasive carcinoma seems to be closely linked to global DNA hypomethylation. Our findings are encouraging and need to be confirmed with a larger cases number study.

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References

Bedford MT, van Helden PD (1987). Hypomethylation of DNA in pathological conditions of the human prostate. *Cancer Res*, **47**, 5274-6.

Burgers WA, Blanchon L, Pradhan S, et al (2007). Viral oncoproteins target the DNA methyltransferases. *Oncogene*, **26**, 1650-5.

Cuschieri K, Wentzensen N (2008). Human papillomavirus mRNA and p16 detection as biomarkers for the improved diagnosis of cervical neoplasia. *Cancer Epidemiol Biomarkers Prev*, **17**, 2536-45.

de Capoa A, Musolino A, Della Rosa S, et al (2003). DNA demethylation is directly related to tumour progression: evidence in normal, pre-malignant and malignant cells from uterine cervix samples. *Oncol Rep*, **10**, 545-9.

Dell G, Gaston K (2001). Human papillomaviruses and their role in cervical cancer. *Cell Mol Life Sci*, **58**, 1923-42.

Ehrlich M, Hopkins NE, Jiang G, et al (2003). Satellite DNA hypomethylation in karyotyped Wilms tumors. *Cancer Genet Cytogenet*, **141**, 97-105.

Ehrlich M (2002). DNA methylation in cancer: too much, but also too little. *Oncogene*, **21**, 5400-13.

Ehrlich M, Jiang G, Fiala E, et al (2002). Hypomethylation and hypermethylation of DNA in Wilms tumors. *Oncogene*, **21**, 6694-702.

Florl AR, Steinhoff C, Muller M, et al (2004). Coordinate hypermethylation at specific genes in prostate carcinoma precedes LINE-1 hypomethylation. *Br J Cancer*, **91**, 985-94.

Fraga MF, Uriol E, Borja Diego L, et al (2002). High-performance capillary electrophoretic method for the quantification of 5-methyl 2'-deoxycytidine in genomic DNA: application to plant, animal and human cancer tissues. *Electrophoresis*, **23**, 1677-81.

Gama-Sosa MA, Slagel VA, Trewyn RW, et al (1983). The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res*, **11**, 6883-94.

Ghabreau L, Roux JP, Niveleau A, et al (2004). Correlation between the DNA global methylation status and progesterone receptor expression in normal endometrium, endometrioid adenocarcinoma and precursors. *Virchows Arch*, **445**, 129-34.

Goyal R, Reinhardt R, Jeltsch A (2006). Accuracy of DNA methylation pattern preservation by the Dnmt1 methyltransferase. *Nucleic Acids Res*, **34**, 1182-8.

Kim YI, Giuliano A, Hatch KD, et al (1994). Global DNA hypomethylation increases progressively in cervical dysplasia and carcinoma. *Cancer*, **74**, 893-9.

Klaes R, Friedrich T, Spitkovsky D, et al (2001). Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer*, **92**, 276-84.

Lin CH, Hsieh SY, Sheen IS, et al (2001). Genome-wide hypomethylation in hepatocellular carcinogenesis. *Cancer Res*, **61**, 4238-43.

Missaoui N, Hmissa S, Frappart L, et al (2006). p16INK4A overexpression and HPV infection in uterine cervix adenocarcinoma. *Virchows Arch*, **448**, 597-603.

Missaoui N, Trabelsi A, Hmissa S, et al (2010). p16INK4A overexpression in precancerous and cancerous lesions of the uterine cervix in Tunisian women. *Pathol Res Pract*, **206**, 550-5.

Ostor AG (1993). Natural history of cervical intraepithelial neoplasia: a critical review. *Int J Gynecol Pathol*, **12**, 186-92.

Parkin DM (2006). The evolution of the population-based cancer registry. *Nat Rev Cancer*, **6**, 603-12.

Perrin D, Ballestar E, Fraga MF, et al (2007). Specific hypermethylation of LINE-1 elements during abnormal overgrowth and differentiation of human placenta. *Oncogene*, **26**, 2518-24.

Piyathilake CJ, Azrad M, Jhala D, et al (2006). Mandatory fortification with folic acid in the United States is not associated with changes in the degree or the pattern of global DNA methylation in cells involved in cervical carcinogenesis. *Cancer Biomark*, **2**, 259-66.

Tavassoli FA, Devilee P (2003). Tumours of the breast and female genital organs. IARC, Lyon

Tost J (2009). DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. *Methods Mol Biol*, **507**, 3-20.

Wahlfors J, Hiltunen H, Heinonen K, et al (1992). Genomic hypomethylation in human chronic lymphocytic leukemia. *Blood*, **80**, 2074-80.

Wentzensen N, Bergeron C, Cas F, et al (2005). Evaluation of a nuclear score for p16INK4a stained cervical squamous cells in liquid based cytology samples. *Cancer*, **105**, 461-7.

Widschwendter M, Jiang G, Woods C, et al (2004). DNA hypomethylation and ovarian cancer biology. *Cancer Res*, **64**, 4472-80.

Yang HJ, Liu VW, Wang Y, et al (2006). Differential DNA methylation profiles in gynecological cancers and correlation with clinico-pathological data. *BMC Cancer*, **6**, 212.

zur Hausen H (2000). Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J Natl Cancer Inst*, **92**, 690-8.

zur Hausen H (2001). Cervical carcinoma and human papillomavirus: on the road to preventing a major human cancer. *J Natl Cancer Inst*, **93**, 252-3.

zur Hausen H (2002). Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer*, 2, 342-50.