
Determination of Endogenous Radioresistance Biomarkers on HeLa Cells

Mamadou Soumboundou^{1, 2, 3, *}, Marie Thérèse Aloy⁴, Macoura Gadji^{5, 6}, Celine Malesys⁴, Gora Mbaye³, Ahmadou Dem⁷, Mounibe Diarra³, Claire Rodriguez-Lafrasse⁴

¹Laboratory of Biophysics, Department of Biology and Functional Explorations, Thies University, Thies, Senegal

²Diagnostic Laboratory, Children Hospital of Diamniadio, Dakar, Senegal

³Laboratory of Pharmaceutical Physics, Pharmacy Department, University Cheikh Anta Diop of Dakar (UCAD), Dakar, Senegal

⁴Molecular and Cellular Radiobiology Laboratory, University of Claude Bernard, Villeurbanne, France

⁵Service of Hematology, National Centre of Blood Transfusion (CNTS), Department of Applied Biological and Pharmaceutical Sciences, Faculty of Medicine, Pharmacy and Odontology (FMPO), University Cheikh Anta Diop of Dakar (UCAD), Dakar, Senegal

⁶Research Institute in Oncology & Haematology (RIOH), Cancer Care Manitoba (CCMB), Department of Physiology & Pathophysiology, University of Manitoba (UFM), Winnipeg, Manitoba, Canada

⁷Cancer Institute, Department of surgery, University Cheikh Anta Diop of Dakar (UCAD), Dakar, Senegal

Email address:

soumboun@hotmail.com (M. Soumboundou), marie-therese.aloy@univ-lyon1.fr (M. T. Aloy), macoura.gadji@ucad.edu.sn (M. Gadji), celine.malesys@univ-lyon1.fr (C. Malesys), mbaygo@yahoo.fr (G. Mbaye), mounbeni@yahoo.fr (M. Diarra), claire.lafrasse-rodriguez@univ-lyon1.fr (C. Rodriguez-Lafrasse)

*Corresponding author

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Abstract: The Genes such as carbonic anhydrase IX (CAIX), hypoxia inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF) have been suggested as hypoxic biomarkers in cancer. Indeed, these endogenous biomarkers have been shown to have stronger prognostic value response after treatment by irradiation. However, inconsistent results suggest that factors other than oxygen influence their expression. This present study deciphers the level of expression of different radioresistance biomarkers in both normoxia and hypoxia conditions followed by irradiation of human ovarian tumor cell lines (uterine cervix squamous cell carcinoma (HeLa). HeLa cells were submitted to hypoxia (1% O₂) conditions in a Thermo Scientific Heracell i CO₂ incubator. The cells were subjected to two doses 4-10 Gy irradiation and re-incubate in their starting conditions for 4 hours, then fixed in 4% paraformaldehyde for 20 min. Protein expressions were assessed by immunocytochemistry staining and fluorescent images were captured by a Axio Imager Z1 fluorescence microscope with oil immersion lens at 63× magnification. In normoxia conditions there was no modification of the level of expression of the CAIX after irradiation. However, an increasing expression level of VEGF was noted. The level of expression of HIF-1 in normoxia was low compared to the other two proteins (CAIX and VEGF). Hypoxia conditions at 2% resulted in a low expression of CAIX and VEGF before and after irradiation at 10 Gy in HeLa cells. HIF-1 had a maximum expression level compared to CAIX and VEGF at 2% oxygen after irradiation in HeLa cells. As tumor hypoxia occurs in a deprived microenvironment, other environmental factors such as irradiation might interact with the effect of low oxygen concentration on gene expression. This study shows that irradiation of HeLa cells has a profound influence on the oxygen dependent induction of certain endogenous hypoxic markers as HIF-1, CAIX, and VEGF.

Keywords: Radioresistance, Biomarkers, Hypoxia, Normoxia, DNA Repair HELA Cells

1. Introduction

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in women worldwide [1].

With over 528,000 new cases and more than 266,000 deaths in 2012 alone, cervical cancer is the fourth most common cancer in women worldwide, and second for women ages 15 to 44 [2]. Roughly 740 deaths per day occur due to cervical cancer, making it the second most common cause of cancer death in women [2].

Approximately 85% of the worldwide deaths from cervical cancer occur in underdeveloped or developing countries, and the death rate is 18 times higher in low income and middle income countries compared with wealthier countries [3].

Radiotherapy (RT) is the first treatment option offered to a majority of patient cases, since most of them present in clinical stages IIB, III or IV [4]. Clinical prognostic factors include tumor stage and nodal involvement, although metabolic response after treatment has been shown to have even stronger prognostic value [5].

A wide variation in the degree of normal tissue injury and tumor response have been observed in patients receiving identical RT. Considerable evidence has emerged that these wide variations in tumor response and normal tissue injury are largely caused by individual differences in intrinsic radiosensitivity [6].

A common feature of most tumors is a low level of oxygen, called hypoxia, the severity of which varies between tumor types [7].

In solid tumors, hypoxia has been proved to be a fundamental pathological feature that exerts numerous effects on the biological behavior of cancer cells. Because of the rapid growth of tumors, cancerous tissues grow beyond the physiological oxygen diffusion limit, and cancer cells activate angiogenesis, a process of generating new blood vessels stemming from the preexisting vasculature [8].

In many malignant tumors, hypoxia microenvironment is associated with poor prognosis, tumor aggressiveness and metastasis, post-treatment recurrence and resistance to radiation therapy [9].

To improve the outcome of cervical cancer, reliable biomarkers can identify patients with aggressive behavior and treatment of resistant tumors are needed [10].

Several endogenous proteins have been suggested as potential biomarkers of tumor hypoxia, including hypoxia-inducible factor-1 α (HIF-1 α , the α subunit of the transcription factor HIF-1) and downstream target genes of HIF-1 such as vascular endothelial growth factor-A (VEGF-A), glucose transporter-1, and carbonic anhydrase IX (CAIX) [11].

HIF-1 α overexpression is associated with an increased risk of mortality in early-stage of cervical carcinoma [12]. Among the target genes of HIF-1, CAIX seems to have the greatest potential as a biomarker of tumor hypoxia in cervical carcinoma. CAIX appears to be strictly regulated by hypoxia via HIF-1 α and shows strong up-regulation in hypoxic regions of tumors of the cervix [13].

Moreover, high level of CAIX in cervical tumors has been shown to be associated with lymph node metastasis and to predict poor overall and metastasis-free survival rates after radiation therapy [14].

An era of personalized cancer medicine in which biomarkers can be used to tailor treatment to each specific patient is a major goal in oncology. Prognostic biomarkers provide information regarding disease outcome regardless of the treatment received and predictive biomarkers determine which patients will probably derive benefit from a specific therapy [15].

The aim of this study was to evaluate the radiobiological response and the level of expression of endogenous radioresistance biomarkers on cervical cancer cell line (HeLa cells using both normoxia and hypoxia conditions followed by irradiation. These results will allow us to search these endogenous radioresistance biomarkers in a series of Senegalese women with cervix cancers attending radiotherapy.

2. Materials and Experiments

2.1. Cell Culture

The cervical cancer HeLa cell line was purchased from the Cell Bank of Laboratory of Cellular and Molecular Radiobiology, EMR3738, Faculty of Medicine Lyon-South (Lyon, France). After thawing, the HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and Glutamax-I (4, 5 g/L D-Glucose) supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100 ug/ml streptomycin and then incubated at 37°C in a humidified atmosphere containing 5% CO₂ incubator (KM-CC17R2, JAPAN). To detach HeLa cells in the logarithmic growth phase, flasks were trypsinized with 1, 5 ml trypsin (Gibco, Thermo Fisher-US, Waltham, Massachusetts) followed by cell counting.

2.2. Reagents and Antibodies

The HIF-1 α antibody (sc-53546), the VEGF mouse monoclonal antibody (sc-7269) and CAIX rabbit polyclonal antibody (sc-25599) were purchased from Santa Cruz Biotechnology, INC 1: 200 (Santa Cruz Biotechnology, Dallas, US).

2.3. Irradiation Technique

After 24 hours of incubation at 37°C, allowing sufficient time for cells to completely adhere to the surface, the cells were treated with radiation in megavoltage radiotherapy machines. Different dose ranging from low dose region (1 Gy to 2 Gy) and beyond, up to 4 Gy were used (6 Gy to 8 Gy). X-ray irradiation was performed at 250 kV delivered at a dose rate of 2 Gy/min using a X-Rad 320 irradiator (Precision X-ray Inc., North Branford, CT).

2.4. Clonogenic Cell Death Assays

Cell survival curves were generated using a standard

colony formation assay. HeLa cells were seeded in 25 cm² culture flasks at densities of 100-1600 cells/cm² to yield 10-200 colonies after irradiation with doses of 1, 2, 4, 6, 8 Gy. After 6 days at 37°C corresponding to six cellular dividing, cells were washed with phosphate buffered saline (PBS), fixed with methanol and stained with Giemsa (Sigma-Aldrich Saint-Louis, Missouri, US). Viable colonies (colonies with cells more than 50) were counted on a Colcount™ (OXFORD Optronix; Nottingham, U.K). In non-irradiated plates (control) percentages of cells seeded that form viable colonies provided plating efficiency (PE). Surviving fraction was calculated using the following formula (1) [16].

$$\frac{\text{Colonies counted}}{\text{Colonies seeded}} \times (P.E/100) \quad (1)$$

Clonogenic survival curves were fitted according to the linear quadratic equation (2); where SF is the surviving fraction, α and β represent the initial slope (probability of lethal events) and the terminal slope (sublethal events) constants respectively; and D represents the irradiation dose. The SF at 2 Gy (SF2) was used as an index of radiosensitivity.

$$SF2 = e^{-[\alpha D + \beta D^2]} \quad (2)$$

2.5. γ -H2AX Immunofluorescence Assay

Phospho-H2AX foci immunofluorescence was detected to monitor the DNA DSBs and DSB repair capacity of HeLa cells. The detection of γ -H2AX foci was assayed by immunocytochemistry. Briefly, cells were fixed in 4% paraformaldehyde for 20 min, and immunodetection was performed as described by Hanot *et al* [17]. Foci were visualized on an Axio Imager Z1 fluorescence microscope (Carl Zeiss S. A. S., Le Pecq, France) and γ -H2AX staining was quantified using Image J software. The results were expressed as mean \pm SD of a minimal counting of 100 nuclei per slide performed in duplicate.

2.6. Endogenous Markers of Hypoxia Induction

Cells (2×10^5) were seeded in six-well plates and cultured until they reached 30% confluence. Cells were additionally cultured in two different conditions for 24 h. In normoxia conditions (control group), cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ incubator (KM-CC17R2, JAPAN); and in hypoxia conditions (hypoxia group) cells were generated in a Thermo Scientific Heracell i CO₂ incubator.

The cells were subjected to two doses 4-10 Gy irradiation and re-incubate in their starting conditions for 4 hours fixed in 4% paraformaldehyde for 20 min.

2.7. Immunocytochemistry Staining

Cells cultured on 15 mm cover slips were washed twice

in PBS 1x and fixed with 4% paraformaldehyde at 4°C for 30 min. After fixation, the cells were permeabilized using 0.1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin for 30 min at room temperature. The samples were then incubated with primary antibodies at 4°C overnight, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Thermo Fisher Scientific, USA) for 1 h at room temperature. The cover slips were then fixed on slides using Fluor shield Mounting Medium with DAPI (Abcam, USA), which contains DAPI for the counterstaining of nuclei. Fluorescent images were captured by an Axio Imager Z1 fluorescence microscope (Carl Zeiss S. A. S., Le Pecq, France) with oil immersion lens at 63 \times magnification.

3. Results

3.1. Clonogenic Cell Death Assays

The results concerning clonogenic cell survival in HeLa cells following X-ray irradiations at 250 KeV are shown in Figure 1. Thus, the survival factor at 2 Gy after exposure to 250 KeV X-ray exposures was 0.48 for HeLa cells. This result shows that HeLa cells are resistant to X-ray irradiation (Figure 1).

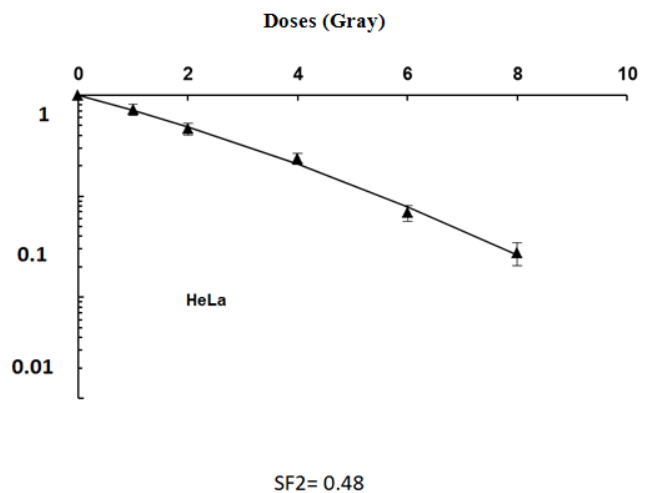


Figure 1. Dose–response curves for cell killing following X-ray exposures. The survival curve was fitted according to linear quadratic equation. Values represent the mean \pm SD of three independent experiments.

3.2. Quantification of γ -H2AX Foci

To confirm the radioresistant character of the HeLa cells, we determined the residual double strand breaks by quantifying the initial (30 min) and residual (24 h) double-strand breaks (DSB) as shown by γ -H2AX foci (Figure 2). HeLa cells show 2.3 ± 0.2 foci /cell at 30 min; exposure to a 2 Gy irradiation induced the formation of 17.9 ± 0.1 foci/cell. As expected 24 h after irradiation, DSBs returned to basal levels given the radioresistance of HeLa cells (Figure 2).

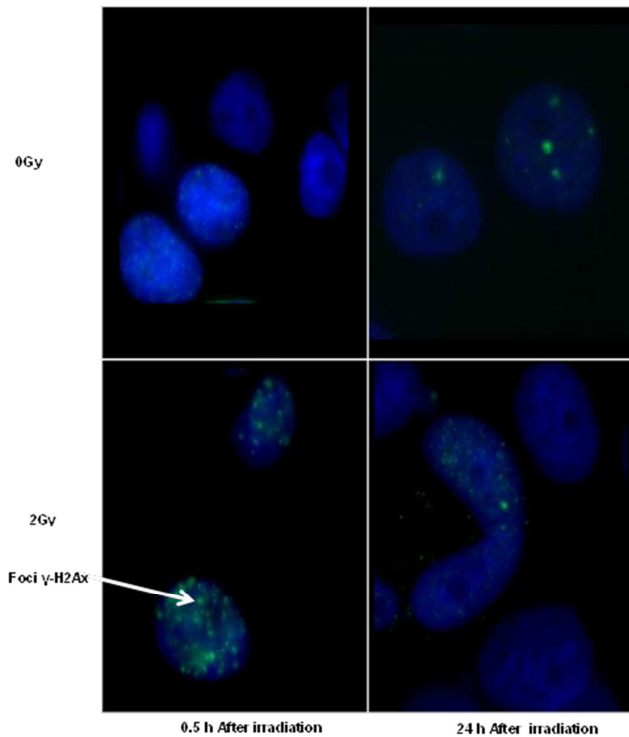


Figure 2. Quantification of γ -H2AX foci 0.5 and 24 h after treatment of HeLa cells with 2Gy irradiation.

3.3. Immunocytochemistry

Qualitative immunocytochemistry analysis showed increased expression of HIF-1 α , CAIX and VEGF at normoxia 24h after X-ray irradiation at 10 Gy compared with none irradiated cells (Figure 3). However, the expression of CAIX was more visible compared to VEGF and HIF-1 α in normoxia conditions (Figure 4).

Normoxia 4h after irradiation 10 Gy

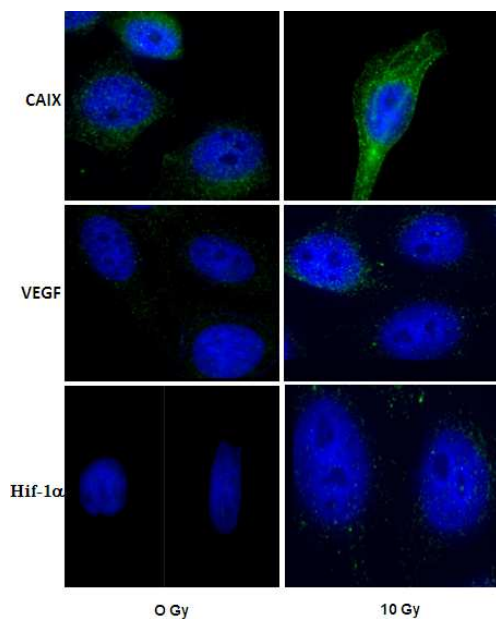


Figure 3. Photograph of an immunofluorescence carried out on a HeLa cells, treated with an anti- Hif-1 α , anti-vegf and CAIX antibody (green proteins) and by the nuclear dye DAPI (blue).

We studied the effect of hypoxia on the level of expression of biomarkers related to its induction in order to answer to this question of whether the level of expression of the different markers could be a function of a physiological condition, namely tumor hypoxia.

Thus, an overexpression of HIF-1 α was noted when the cells were maintained in hypoxia (24h in hypoxia 1% O₂, Irradiation 10 Gy, 4h in Hypoxia 1% O₂). However, the level of expressions of CAIX and VEGF was not microscopically significant compared to expression in normoxia conditions (Figure 3).

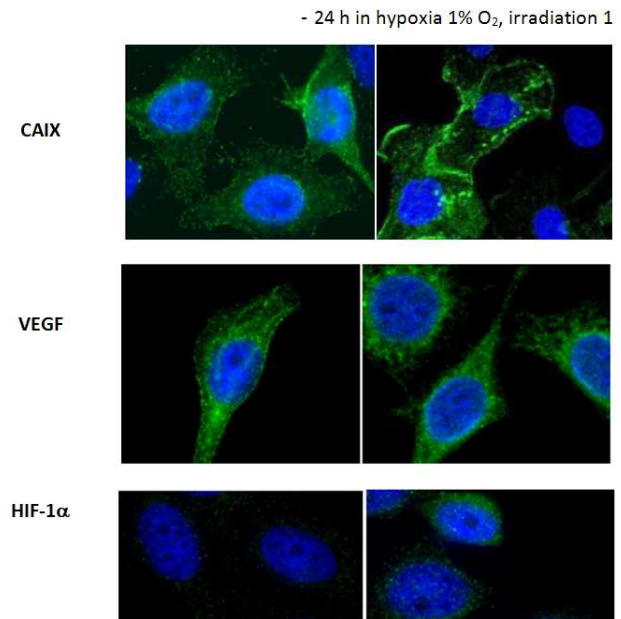


Figure 4. Photograph of an immunofluorescence carried out on a HeLa cells, treated with an anti- Hif-1 α , anti-vegf and CAIX antibody (green proteins) and by the nuclear dye DAPI (blue).

4. Discussion

Tumor hypoxia represents a significant obstacle in the clinical management of locally advanced carcinoma of the uterine cervix, by increasing malignancy and inducing treatment resistance [11].

A major player in the hypoxia response is HIF-1 α , with its downstream target genes involved in cell survival, metabolism, angiogenesis, and metastasis [18].

Based on a number of clinical studies, several investigators have promoted the view that the expression of HIF-1 α and its target genes may be useful biomarkers of tumor hypoxia, aggressiveness, and radiation resistance in cervical carcinoma [19]. In this present preclinical study, we challenged this view by searching correlations between tumor hypoxia and the expression of HIF-1 α , VEGF and CAIX in HeLa cervical carcinoma cells lines.

A significant advantage of preclinical investigations over clinical investigations is the measurement conditions can be kept unchanged during the clinical study. A major disadvantage of preclinical studies is experimental cells may not be necessary valid models of human cancer.

Radiotherapy is still one of the best cervical cancer therapy. Radiotherapy technology has evolved remarkably during the past decade, and radiation can be precisely delivered, thereby allowing higher doses to the tumor and reducing doses to surrounding normal tissues [20].

Tumor recurrence following a course of radiation therapy results from radiation resistance in some cells which can survive and re-proliferate. One of the major reasons for the failure of radiation therapy is hypoxia [21, 22].

It is well known that HIF-1 α promotes tumor radioresistance through stimulation of endothelial cell survival [23]. HIF-1 α expression is associated with a reduced survival in a variety of human cancers including uterine cervix [24], ovary [25], esophagus, lung, and breast and may also influence resistance to therapy in several cancer types [26].

Potential explanations for these differences include tumors heterogeneity in the level of oxygenation and hence HIF-1 α expression; and HIF-1 α is rapidly degraded with restoration of normoxia, making HIF-1 α more reflective of acute rather than chronic tumor hypoxia [27].

Of all the considered biomarkers in this preclinical study, HIF-1 α , VEGF, CAIX proteins showed significant findings in hypoxia after irradiation but not in normoxia. Indeed, in normoxia conditions the low expression of HIF-1 α found can be explained by the fact HIF-1 α 's rapid degradation with restoration of normoxia, making HIF-1 α more reflective of acute rather than chronic tumor hypoxia [27].

That is why; the data suggest that for each cancer it will be important to determine whether HIF-1 α overexpression, either alone or in combination with other markers such as p53 or BCL2, can identify a subset of patients with more aggressive disease for whom standard therapy will not be sufficient to ensure a high probability of survival.

Additionally, the transiently expression of HIF-1 α compared with metabolic proteins expression, resulting in increased potential false-negative results from HIF-1 α staining.

Our results have shown that CAIX and VEGF are overexpressed on radioresistant HeLa cells line in both conditions 63 \times magnification. normoxia and hypoxia.

In fact, several studies have demonstrated that high CAIX and VEGF expression level in tumor cells correlate with greater malignant potential, more aggressive growth characteristics and risk of metastasis [28, 29] 63 \times magnification [30].

This result compared to the level of expression of the CAIX proves its usefulness in medical imaging because it is not depressed by the physiological conditions. Pastorkova *et al.*, 2008 had already mentioned the usefulness of CAIX as an emerging target for cancer therapy and functional imaging [31], furthermore broadening the potential clinical value of the CAIX biomarker in the detection, treatment and management of women with cervical cancer.

5. Conclusion

In radiotherapy, the treatment is adapted to each individual to protect healthy tissues but delivers most of time a standard

dose according to the tumor histology and site. The only biomarkers studied to individualize the treatment are the HPV status with radiation dose de-escalation strategies, and tumor hypoxia with dose escalation to hypoxic subvolumes using FMISO- or FAZA-PET imaging.

These preclinical results on the radio-resistant HeLa cells show different level of expression of radioresistant proteins such as HIF-1 α , CAIX and VEGF. Thus, the development of prognostic index according to the different biomarkers is feasible by immunohistochemistry on human cervical cancer biopsies. These different biomarkers would allow better selection and follow-up of patients bearing cervical cancer, and an integration of the intrinsic biological data of the tumor in the treatment.

Finally, the systematic search for potential biomarkers predicting the response to irradiation could be used to modulate therapy in the event of a positive response biomarker, or, conversely, to stop, as part of a treatment associated with the treatment irradiation.

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Conflict of Interest

The authors have no conflict of interest to disclose.

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