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Immunofluorescence Analysis of Erythrocyte Membranes of Cervical Cancer Patients

Sargylana N. Mamaeva[1], Vladislav A. Alekseev[2], Nadezhda A. Nikolaeva[1], Tatyana A. Krylova[1], Aya A. Gabysheva[1], Alexandr N. Pavlov[1], Irina V. Kononova[2], Georgy V. Maksimov[3]

[1]*M.K. Ammosov North-Eastern Federal University, Yakutsk, Sakha (Yakutia) Republic, Russia*
[2]*Yakut Scientific Center of Complex Medical Problems, Yakutsk, Sakha (Yakutia) Republic, Russia*
[3]*Lomonosov Moscow State University, Moscow, Russia*

Abstract

Currently, cervical cancer (CC) is one of the most common oncological diseases. In this regard, it is necessary to develop new research methods for a more detailed study of the occurrence and development of the disease at the molecular and cellular levels, as well as to improve the effectiveness of treatment and form a deeper understanding of the causes of relapses. The aim of this work was to study nanoparticles localized on the erythrocyte membrane—presumably HPV 16, 18, before and after radiation therapy, in patients with CC.

To study the surface of red blood cells by the SEM method, venous blood samples from 17 patients with a confirmed diagnosis of CC were prepared in thin layers evenly applied to a dry, fat-free glass slide, which was dried at room temperature. To detect nanoparticles on the surface of erythrocytes by the immunofluorescence assay, we developed a special protocol for preparing erythrocyte masses from patients diagnosed with CC. As a result of using a new method of sample preparation for immunofluorescence assay and using SEM, the hypothesis of the viral nature of nanoparticles localized on the surface of the blood erythrocytes of patients with CC was confirmed: Particles of HPV 16 and 18 are located on the cytoplasmic membrane of erythrocytes. Studies suggest that viruses attach to the erythrocyte membrane, which seems to influence the development of CC, its recurrence, and metastasis.(**International Journal of Biomedicine. 2023;13(1):69-72.**)

Keywords: cervical cancer • immunofluorescence assay • erythrocyte • HPV • scanning electron microscope

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Abbreviations

CC, cervical cancer; **HPV**, human papillomavirus; **IFA**, immunofluorescence assay; **SEM**, scanning electron microscope

Introduction

Currently, cervical cancer (CC) is one of the most common oncological diseases. Epidemiological and virological studies have revealed that 95% of all squamous cell cancers of the cervix are caused by human papillomavirus (HPV) and contain HPV-DNA 16,18. By the end of 2020, according to the International Agency for Research on Cancer GLOBOCAN, CC was among the top 10 cancers (using estimates of morbidity and mortality). In 2020, 604,127 new cases and 341,831 deaths were registered.[1,2]

An even more significant increase in the incidence of CC is expected despite the development of new comprehensive prevention measures to control CC, methods of early diagnosis, and the development of high-tech therapy. In addition, there are frequent recurrences of

CC after various types of radical treatment, including after radiation therapy in the early stages of the disease and the phenomenon of metastasis, which complicates therapy and affects the survival of patients.[3-8]

Note that now there is no clear explanation for the recurrence of this disease. In this regard, it is necessary to develop new research methods for a more detailed study of the occurrence and development of the disease at the molecular and cellular levels, as well as to improve the effectiveness of treatment and form a deeper understanding of the causes of relapses.

According to the results of studies of red blood cells from CC patients using scanning electron microscopy (SEM), obtained by the authors previously, nanoparticles were found on the surface of erythrocytes, the sizes of which were comparable to those of HPV16, 18, and other viruses.[9] In this regard, it became necessary to identify these nanoparticles, determine their nature, and explain the dependence of erythrocyte morphology on radiation therapy. It has been suggested that nanoparticles localized on the surface of erythrocytes before, during, and after radiation therapy could be viruses and vesicles.[10]

In addition, the authors previously established the dependence of these particles' number, morphology, and size on the radiation therapy stages. The appearance of a large number of nanoparticles during radiation therapy and their change (up to complete disappearance at the end of therapy) in the blood (both on erythrocytes and in plasma)—the sizes of which were determined as continuous values, in contrast to nanoparticles that were detected before radiation therapy, the sizes of which were determined as discrete values—can be considered as factors in the reaction of the human body to exposure to ionizing radiation during radiation therapy. In some patients (with stages 3 and 4 of cancer), the presence of membrane-bound nanoparticles is probably due to structures on the erythrocyte surface that existed even before radiation therapy. These nanoparticles are likely viruses. Both viruses that remain in the blood after therapy and vesicles localized on the surface of the erythrocyte membrane may be provocative factors for disease relapses. The appearance of a large number of vesicles in the blood during radiation therapy may be associated, in particular, with altered properties of the membranes of dysmorphic erythrocytes.[10]

The aim of this work was to study nanoparticles localized on the erythrocyte membrane—presumably HPV 16, 18, before and after radiation therapy, in patients with CC. The results of such a study will allow us in the future to evaluate the immunological role of erythrocytes in the development of the disease.

Materials and Methods

This study used venous blood samples from 17 patients with a confirmed diagnosis of CC before and after radiation therapy. The scheme of radiation therapy was described in detail in our previous work.[10]

To study the surface of red blood cells by the SEM method, venous blood samples were prepared in thin layers evenly applied to a dry, fat-free glass slide, which was dried at room temperature. To detect nanoparticles on the surface of erythrocytes by the immunofluorescence analysis, we developed a special protocol for preparing erythrocyte masses from patients diagnosed with CC.

Sample preparation

Blood samples collected in tubes containing EDTA were centrifuged for 5 minutes at 600g. The supernatant was drained, and the precipitate was placed in a 15 ml centrifuge tube for further washing with a phosphate buffer (PBS). We mainly took the central part of the sediment without touching the upper boundary of the phases and the bottom of the tube, where various nucleated cells and other blood elements were concentrated. After the transfer of the erythrocyte fraction, the total volume of the suspension was brought to 10ml using PBS, after which the samples were centrifuged for 5 minutes at 600g. The phosphate buffer washing procedure was repeated 3-4 times. The cells were fixed with 1% paraformaldehyde solution in PBS. A fixing solution was added to the washed sediment, bringing the total volume to 10ml, and then gently mixed by turning the test tube several times and incubating for 15 minutes at room temperature. The fixing solution was removed by centrifugation under the same conditions, and the precipitate was washed 2 times with FACS solution (2% veal serum in PBS). At this stage, the samples were ready for immunofluorescence analysis.

Immunofluorescence assay

This method is widely used in modern cellular and molecular biology and is described in sufficient detail in many works. In our case, the suspension of a fixed sample was diluted with a FACS solution in a ratio of 1:3 (250:750 µl, respectively); then 100 µl of the diluted sample was transferred to a clean Eppendorf-type tube, and 1µl of primary antibodies was carefully added, followed by incubation overnight at +4 °C. Mouse monoclonal antibodies against HPV protein types 1, 6, 11, 16, 18, and 31 MAB837 (Sigma-Aldrich) were used as primary antibodies. After incubation, the sample was centrifuged for 5 minutes at 800 g; the supernatant was drained and washed 3 times with FACS solution. To the sample dissolved in 500 µl of FACS solution was added 2 µl of secondary antibodies, which were goat polyclonal antibodies against mouse immunoglobulin H and L chains conjugated with fluorescein (Stemcell Technologies, cat. # 60138FI).

Dry smears were prepared after an hour of incubation of samples with antibodies and visualized using an AxioVert.A1 microscope with a FITC fluorescent filter.

Scanning electron microscopy

SEM was used to investigate the morphology and surface of red blood cells in CC patients before and after radiation therapy. A high-resolution SEM JSM-7800F (Japanese Electron Optics Laboratory, JEOL, Japan) equipped with a Schottky thermal field emission cathode, a super hybrid objective lens, and a Gentle Beam system was used. The smears were examined at an accelerating voltage of 1.3 kV and a focal length of 4.0 mm using the Gentle Beam system.

The study was conducted in accordance with ethical principles of the WMA Declaration of Helsinki (1964, ed. 2013) and approved by the Ethics Committee of the M.K. Ammosov North-Eastern Federal University (protocol No. 13 of April 4, 2018, decision No. 2). Written informed consent was obtained from each patient.

Results

In this work, in parallel, examinations of the same samples of whole venous blood of CC patients were carried out using different methods: 1) study of the morphology of erythrocytes of dry smears using the SEM method; 2) study of erythrocytes by the MFA method.

Figure 1 shows an SEM image of the red blood cell surface of a dry blood smear of a CC patient. Nanoparticles were detected on erythrocytes, the sizes of which are comparable to those of HPV 16,18.

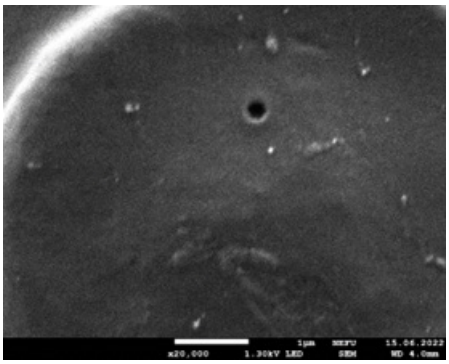

Fig. 1. *SEM image of the erythrocyte surface at 20,000x*

Figure 2 (before radiation therapy) shows images of erythrocytes of the erythrocyte mass obtained based on a new protocol we developed for sample preparation in a CC patient.

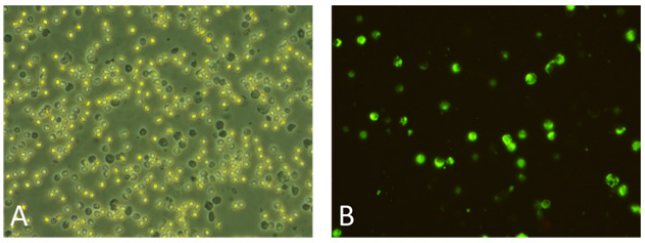

Fig. 2. *Micrographs of erythrocytes of a patient with cervical cancer (400x). (A) phase contrast; (B) fluorescent mode.*

These images were obtained using a fluorescent microscope at various magnifications and microphotography conditions: with phase contrast and in fluorescent mode. The images show a glow on the surface of some red blood cells, thereby confirming the authors' assumptions that the nanoparticles observed in the SEM images may be viral particles.

To detect the nucleus-containing cells, the samples were also stained with Hoechst 33342 dye; however, we did not detect glowing nuclei in the ultraviolet spectrum, which indicates the luminous elements we detected are erythrocytes. In addition, it was found that the luminescence on the surface of erythrocytes is observed in patient samples before and after radiation therapy, i.e., these particles were present even before radiation therapy and were in no way associated with exposure to ionizing radiation.

As a result of using a new method of sample preparation for immunofluorescence analysis and using SEM, the hypothesis of the viral nature of nanoparticles localized on the surface of the blood erythrocytes of patients with CC was confirmed: Particles of HPV 16 and 18 are located on the cytoplasmic membrane of erythrocytes.

Studies suggest that viruses attach to the erythrocyte membrane, which seems to influence the development of CC, its recurrence, and metastasis.

Discussion

Our previous studies lacked sufficient results in favor of identifying nanoparticles on the surface of erythrocytes as viruses. The results of this study are an additional link in the definition of these nanoparticles as viruses. Another group of researchers has shown that surface receptors and ligands of exosomes are responsible for the distribution and attachment of exosomes to target cells and extracellular matrix.[11,12] Consequently, exosomes circulating in the blood and attached to blood cells do not undergo immediate fusion with cells but remain exosomes attached to the cell surface for some time. The role of circulating exosomes attached to the surface of erythrocytes in the spread of the tumor remains unclear; however, convincing evidence has been obtained that RNA and proteins included in such particles can play an important role in the diagnosis of cancer.[13,14]

Based on the results of this work and that of other researchers, it can be argued that the HPV can attach and be transported to the surface of red blood cells, affecting their morphology and biophysical properties, possibly playing a decisive role in the development of the disease, and have an impact on the results of therapy and relapses of the disease.

Significant evidence has also recently been obtained indicating that HPV may play a role in developing CC. However, the data linking CC with chronic HPV infection were contradictory, which led to a lack of consensus.[15] We plan to conduct more focused research to confirm the results of our study.

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Competing Interests

The authors declare that they have no competing interests.

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***Corresponding author**: Sargylana Mamaeva, Ph.D. M.K. Ammosov North-Eastern Federal University, Yakutsk, Sakha (Yakutia) Republic, Russia. **E-mail**: sargylana_mamaeva@mail.ru*