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Chapter

Study of the Fluorescence Intensity of Histones HP1 γ , H3K9me₃, H3K27me₃, and Histone Deacetylases SIRT1 and SIRT6 in Dermal Fibroblasts of Patients with Breast Cancer and Patients at Risk of Cancer

Mirya Kuranova, Aleksandra Nozdracheva, Nadezhda Pleskach, Daria Pasekova and Aleksey Manikhas

Abstract

Breast cancer is by far the most common cancer among women. The heterogeneous nature of the onset of this disease has already been proven and a search is underway for possible epigenetic mechanisms that affect the course of treatment. The indirect immunofluorescence method was used to study the fluorescence intensity of histones HP1 γ , H3K9me₃, H3K27me₃, and histone deacetylases SIRT1 and SIRT6 in dermal fibroblasts of patients with breast cancer and patients at risk of developing cancer. The results of the study showed a significant difference in the level of fluorescence intensity of histones H3K9me₃ and H3K27me₃ in the cells of patients with breast cancer from the values in the cells of healthy donors. Also, the results of the study suggest that the heterochromatin protein HP1 γ is a possible marker of the heterogeneity of breast cancer.

Keywords: breast cancer, immunofluorescence, HP1 γ , H3K9me₃, H3K27me₃, SIRT1, SIRT6

1. Introduction

Despite significant advances in cancer research, breast cancer remains a serious problem and, according to WHO, is the most common cancer worldwide affecting women. It accounts for 16% of all oncological diseases in women with incidence rates of 99.4 per 100,000 women aged 13–90 years. According to forecasts in the coming years, its frequency and mortality will increase significantly.

Modern medicine shows that progress in the fight against breast cancer over the past decade is slow. This is not surprising given the important limitations of currently available targeted treatments. The reasons for the high internal and acquired resistance to available target drugs include their temporary antitumor activity and the lack of consideration for origins of heterogeneity of tumors. Understanding this extremely complex heterogeneity is crucial in the “war” against breast cancer and metastasis.

Oncogene activation in mammalian cells leads to proliferative stress and the induction of aging, which limits tumor growth. Thus, aging is the physiological mechanism of tumor suppression, which prevents progression from benign tumor foci to malignant tumors. Induction of aging by oncogene activation is called oncogene-induced senescence, OIS. Oncogene inactivation, on the other hand, can also cause aging. MYC inactivation causes cell aging, and regression in various tumor samples, such as lymphoma, osteosarcoma, and hepatocellular carcinoma (HCC) [1].

Recently, researchers have been interested in breast cancer in young women. Current evidence suggests that in women under the age of 45, breast cancer is undoubtedly the main cause of cancer mortality. This type of cancer is most likely very heterogeneous and has potentially aggressive and complex biological features. However, management strategies, recommendations, and options are not based on age, and the “complex” biology of this type of cancer remains uncertain and unexplored [2].

Recent studies suggest a significant association between biological aging and objective cognitive performance in breast cancer survivors. Future prospective studies are needed to confirm the causative role of biological aging as a driver of cognitive decline after cancer treatment [3].

To study the processes of activation of cell aging in tumor cells, it is important to understand the effect of chromatin structure on cell proliferation, aging of cells and tissues, and the treatment of cancer. The foci of heterochromatin associated with aging (SAHF) are specialized domains of facultative heterochromatin that form in aging human cells. Although SAHFs are highly densified domains of heterochromatin, they largely exclude other chromatin domains in telomeres and pericentromeres, which are themselves believed to be heterochromatic.

HP1 proteins are important components of a dynamic nuclear response that detects and eliminates defects in the epigenetic information encoded in chromatin through histone modifications and DNA methylation. Defects in this “chromatin repair” response in transformed cells can contribute to the preferential killing of cancer cells using cancer epigenetic therapy, which is still under clinical development [4].

In breast cancer, the level of the H3K27me3 protein rises to maintain the LOX gene in a repressed state [5]. The global level of H3K9me3 protein, on the contrary, decreases during transformation, and its distribution over chromatin is reprogrammed. Perhaps, this is due to the mechanism of the occurrence of breast cancer by regulating gene expression and chromatin stability during cell transformation [6]. Epigenetic modifications of the H3K9me3 and H3K27me3 histones may be useful as diagnostic/prognostic biomarkers and/or therapeutic targets for breast cancer, since DNA and histone modifications are involved in the activation of PD-1, CTLA-4, TIM-3, and LAG-3 in tissue of breast tumors [7, 8].

The intratumoral heterogeneity due to the presence of cancer stem cells (cancer stem cell [CSC]) is the main cause of drug resistance and distant metastases of breast cancer. Histone deacetylase SIRT1 outlines a central role in the regulation of CSC and the SIRT1 pathway can be a promising therapeutic

strategy [9, 10]. Overexpression of SIRT1 significantly stimulates breast cancer growth both in vitro and in vivo, while SIRT1 knockdown inhibits cancer phenotypes [11]. Also, histone deacetylase SIRT6 may be a therapeutic marker. Sirtuin 6 (SIRT6) is associated with longevity and is also a tumor suppressor. The survival of patients with breast cancer was positively correlated with the abundance of SIRT6 and inversely correlated with phosphorylation of SIRT6 at Ser (338) [12]. SIRT1 and SIRT6 play a role in fine-tuning the acetylation of the antitumor suppressor FOXO3 (Forkhead Box O3) and sensitivity to lapatinib. The results of immunohistochemistry for different subtypes of breast cancer showed that the high levels of SIRT6/1 are associated with constant high expression of FOXO3, which in turn is associated with deregulation/inactivation of FOXO3 and a poor prognosis of the course of the disease [13].

2. Material and methods

2.1 Study group selection

We studied three groups:

1. Patients with breast cancer: two patients 30 (BC30SP1) and 55 (BC55SP1) years old.
2. Breast cancer risk group: a 30-year-old person with 5382insC mutation in the BRCA1 gene (BRCA1SP) and two mothers of patients with ataxia-telangiectasia—47 (AT8SP) and 45 (AT9SP) years old. Heterozygous carriers of mutations in the ATM gene are at risk of tumors, including breast cancer [14].
3. Healthy donors: two donors without breast cancer or a history of oncological diseases of 30 (N9SP) and 55 (FK19) years old.

2.2 Cell line production

Cell lines of dermal fibroblasts were obtained by the migration method from post-operative material. Lines of dermal fibroblasts BRCA1SP, BC30SP1, and BC55SP1 were obtained from a skin biopsy, which was performed under local anesthesia. Skin biopsies were placed in Petri dishes, filled with complete MEM growth medium (Gibco, USA) containing 15% bovine fetal serum (Gibco, USA), 100 IU/ml penicillin, 100 μ g/ml streptomycin (Gibco, USA), 25 μ g/ml fungicon, 0.3 mg/ml L-glutamine, and cultured in a CO₂ incubator at +37°C in an atmosphere with 5% CO₂ until a subconfluent monolayer was obtained. Next, the cells were rinsed with a solution of phosphate-buffered saline (PBS), removed from Petri dishes using 0.25% Trypsin-Versene solution (Gibco, USA) and scattered on coverslips (Carl Zeiss, Germany).

2.3 Immunocytochemical analysis

By indirect immunofluorescence method, the cell lines were stained with antibodies to chromatin proteins HP1 γ , H3K9me3, H3K27me3, and histone deacetylases SIRT1 and SIRT6.

According to the standard protocol and usage annotations of antibodies, the cells were fixed on ice with a 4% formaldehyde solution for 10 minutes at +40°C followed by fixation with ice-cold 70% ethanol solution in PBS (15 min at room temperature), washed with a solution of PBS, permeabilized with a 1.5% X-100 triton solution in PBS (15 min at room temperature), washed with a PBS solution 3 times for 5 minutes, and incubated in a blocking solution of 5% BSA in PBS (1 h at room temperature), followed by washing with PBS. As the first antibodies, mouse IgG to H3K9me3, H3K27me3, and SIRT1 (Cell Signaling, UK) and rabbit IgG to the HP1 γ protein (Abcam, UK), H3K27me3, SIRT6, and SIRT7 (Cell Signaling, UK) were used. Goat antibodies against mouse or rabbit IgG conjugated with Alexa Fluor 488 and 546 (Invitrogen, USA) at a 1: 500 dilution were used as the second antibodies. To prevent the rapid burnout of immunofluorescence dyes, the preparations were enclosed in antifading using SlowFade Gold Antifade reagent with DAPI (Invitrogen, USA).

2.4 Microscopy

Images from stained preparations were obtained using an AXIOVERT200-DFC420 fluorescent inverted microscope (Carl Zeiss AG, Germany) equipped with a Leica DFC 420 camera and an E \times 40/0.75 lens. In each line, using the Fiji program, fluorescence intensity in 100 cells was determined.

2.5 Statistical analysis

For the parameter of statistical analysis, the standard deviation of the mean values was chosen. Statistical analysis was performed in Google Sheets and Excel.

3. Results

The fluorescence intensity of chromatin proteins HP1 γ , H3K9me3, H3K27me3, and histone deacetylases SIRT1 and SIRT6 was measured in the studied lines (**Figure 1**).

During the fluorescence intensity analysis, we studied both average intensity and intensity distribution. Intensity distribution in all cell lines was visualized with histograms plotted in Google Sheets in two ways:

- with automatic bin size selection
- with manual bin size selection—5 RFU, chosen empirically.

Histograms of the distribution of fluorescence intensities make it possible to assess how heterogeneous the cell line is for the studied protein and also to detect the presence of several subpopulations in cell lines.

Overlapping histograms of the fluorescence intensities of different cell lines with the same bin size allows comparing the distribution of the same protein in different cell lines.

3.1 HP1 γ fluorescence intensity

The highest value of the average HP1 γ fluorescence intensity was detected in dermal fibroblasts of the 30-year-old patient with breast cancer, and it was 8.25 times higher than that of the 55-year-old patient with breast cancer, 3.5

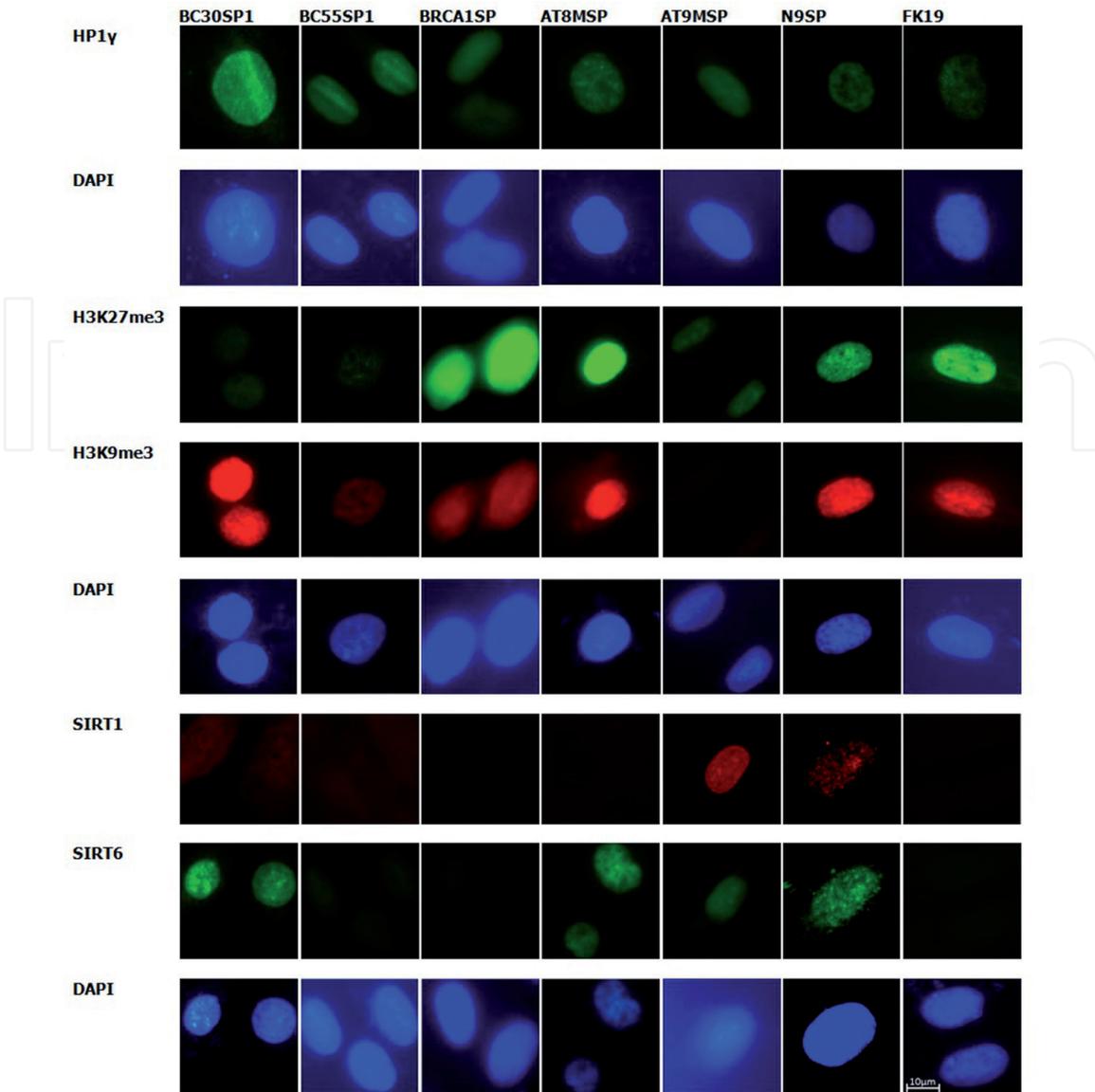


Figure 1.
 Immunocytochemical analysis of the studied lines.

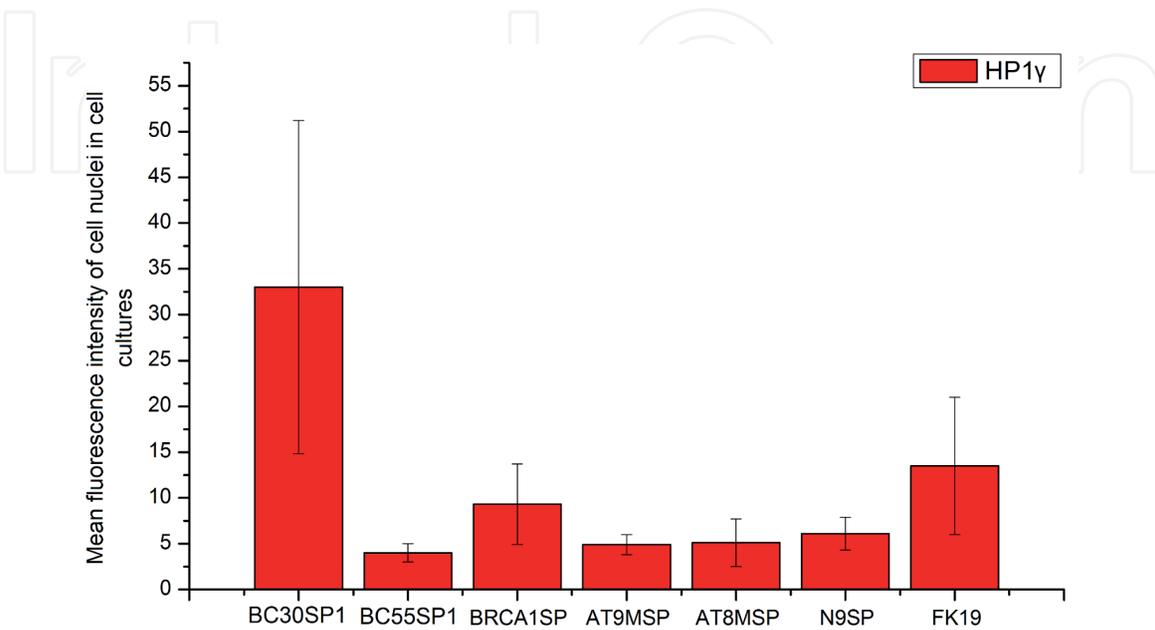


Figure 2.
 Average fluorescence intensity of HP1 γ in the nuclei of dermal fibroblasts of the studied lines.

times more than that of a patient at risk with a mutation in the *BRCA1* gene—BRCA1SP; 6.7 and 6.3 times more than mothers of patients with AT (AT9SP and AT8SP) (**Figure 2**). Such difference in the values of HP1 γ fluorescence intensity can be explained by the fact that the histone heterochromatin protein HP1 γ is considered a marker of aging and its amount decreases in the cell with age. In addition, the nature of the cancer of a 30-year-old patient, most probably, is different than that of a 55-year-old patient.

The average fluorescence intensity levels of HP1 γ in healthy donors did not significantly differ from each other, despite the fact that the data differed by a factor of 6.1 and 13.5 RFU in lines N9SP and FK19, respectively. A significant difference in the average HP1 γ fluorescence intensity of the BS30SP1 line was observed only in relation to the line of one healthy N9SP donor and was 5.4 times greater.

The absence of a significant difference between the cells of healthy donors is explained by the large value of the standard deviation in the population of lines due to the large spread of data.

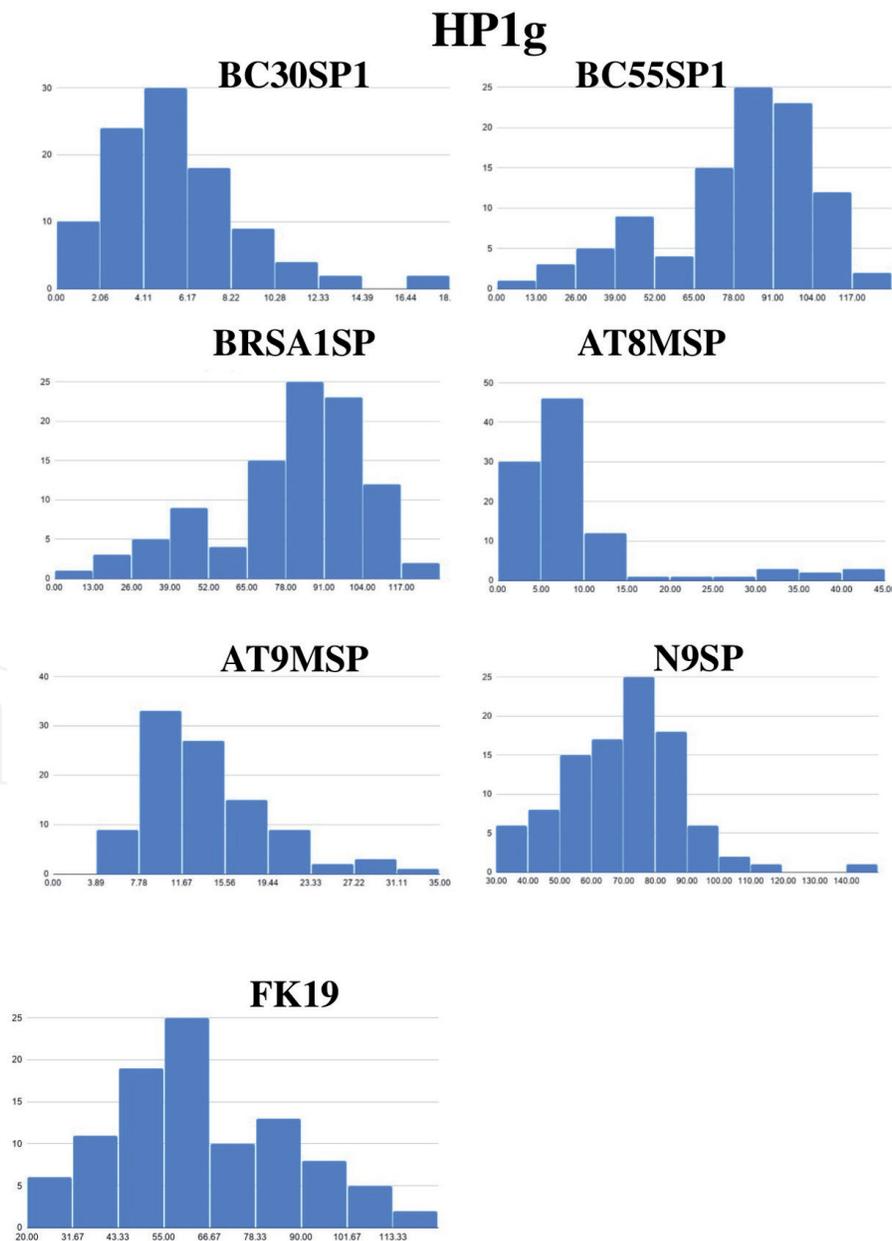


Figure 3.
Histograms of fluorescence intensity distribution of HP1 γ .

The histogram of the distribution of HP1 γ fluorescence intensities (**Figure 3**) in the cell lines showed the presence of two subpopulations in the BC55SP1 and BRCA1SP lines, each with its own distribution. In the 55-year-old healthy donor line, the boundary between the two subpopulations was less pronounced. Perhaps, this is due to age-related epigenetic changes in the HP1 γ protein. In the remaining lines, insignificant second intensity peaks in some cells were observed, which may be associated with some rare cell changes. Interestingly, the picture of the distribution of HP1 γ fluorescence intensity in patients with breast cancer differed from each other, as did the values of the average intensity.

When overlapping the histograms of HP1 γ fluorescence intensity distribution in different cell lines with the same bin size, one can see how widely the HP1 γ

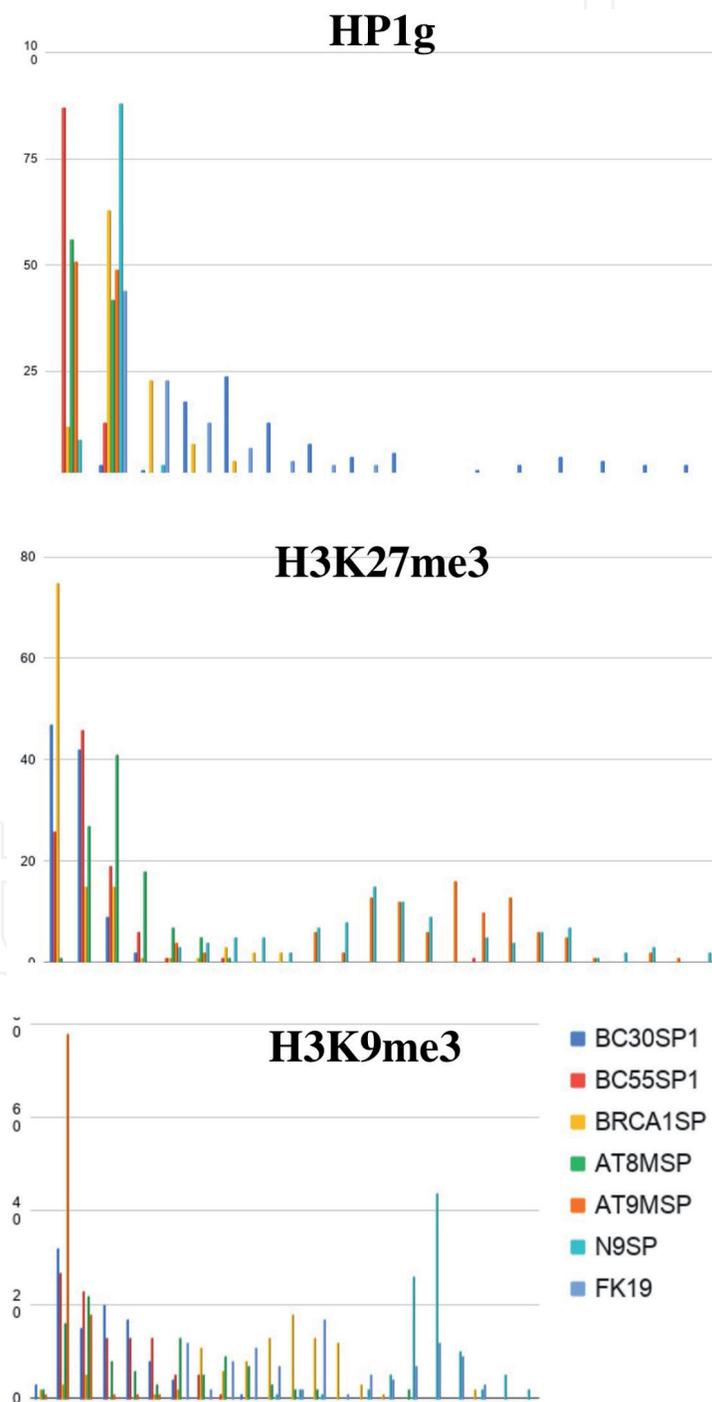


Figure 4. Overlapping histograms of the distribution of fluorescence intensities of HP1 γ , H3K27me3, and H3K9me3 in the studied lines with bin size of 5 RFU.

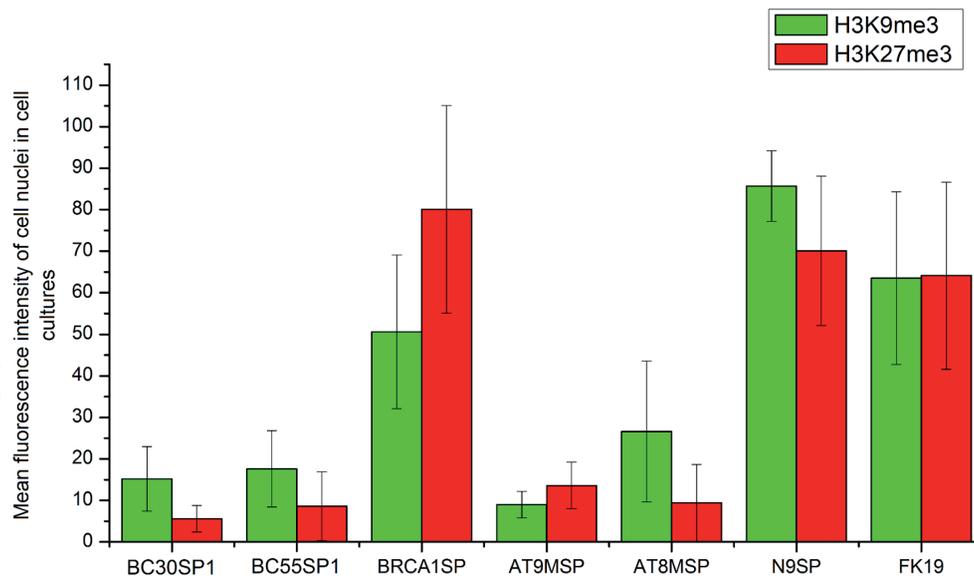


Figure 5. Average fluorescence intensity of H3K27me3 and H3K9me3 in the nuclei of dermal fibroblasts of the studied lines.

fluorescence intensities were distributed in the BC30SP1 line, in contrast to other lines (Figure 4).

3.2 Fluorescence intensity of H3K9me3 and H3K27me3

The average fluorescence intensity levels of H3K9me3 and H3K27me3 in the dermal fibroblast lines of patients with breast cancer significantly differed from the levels in the cells of healthy donors (Figure 5). The average levels of H3K9me3 fluorescence intensity in the dermal fibroblast lines BC30SP1 and BC55SP1 virtually did not differ from each other (15.2 and 17.6 RFU) and were significantly lower by 5 and 4 times than the values of healthy donors 30 and 55 years old, respectively, for which these indicators were the highest. Interestingly, in the cells of the BRCA1 gene mutation's carrier, the average value of the H3K9me3 fluorescence intensity was significantly 3 times higher than in the cells of patients with breast cancer, although the data in the cells of mothers of patients with AT did not significantly differ from patients with breast cancer.

Histograms of the distribution of fluorescence intensities of H3K27me3 and H3K9me3 did not reveal two cell subpopulations in any of the studied lines. In all lines, insignificant second peaks of fluorescence intensities were observed (Figures 6 and 7). The distribution of fluorescence intensities of H3K27me3 and H3K9me3 revealed something similar to two subpopulations, but with very blurred boundaries. This picture may possibly be associated with age-related epigenetic changes in the cell.

When overlapping the histograms of H3K27me3 and H3K9me3 fluorescence intensity distribution in different cell lines with the same bin size, it can be seen that all lines have a sufficiently wide range of fluorescence intensities of H3K27me3 and H3K9me3, except for the BC30SP1 line, which has a fairly narrow range of fluorescence intensities of H3K27me3. This fact suggests a greater sensitivity of H3K27me3 and H3K9me3 histones to the state of the cell (cell cycle, apoptosis, ptosis, etc.). The widest distribution of fluorescence intensities was shown in the BRSA1SP, AT8MSP, and N9SP lines (Figure 4).

H3K27me3

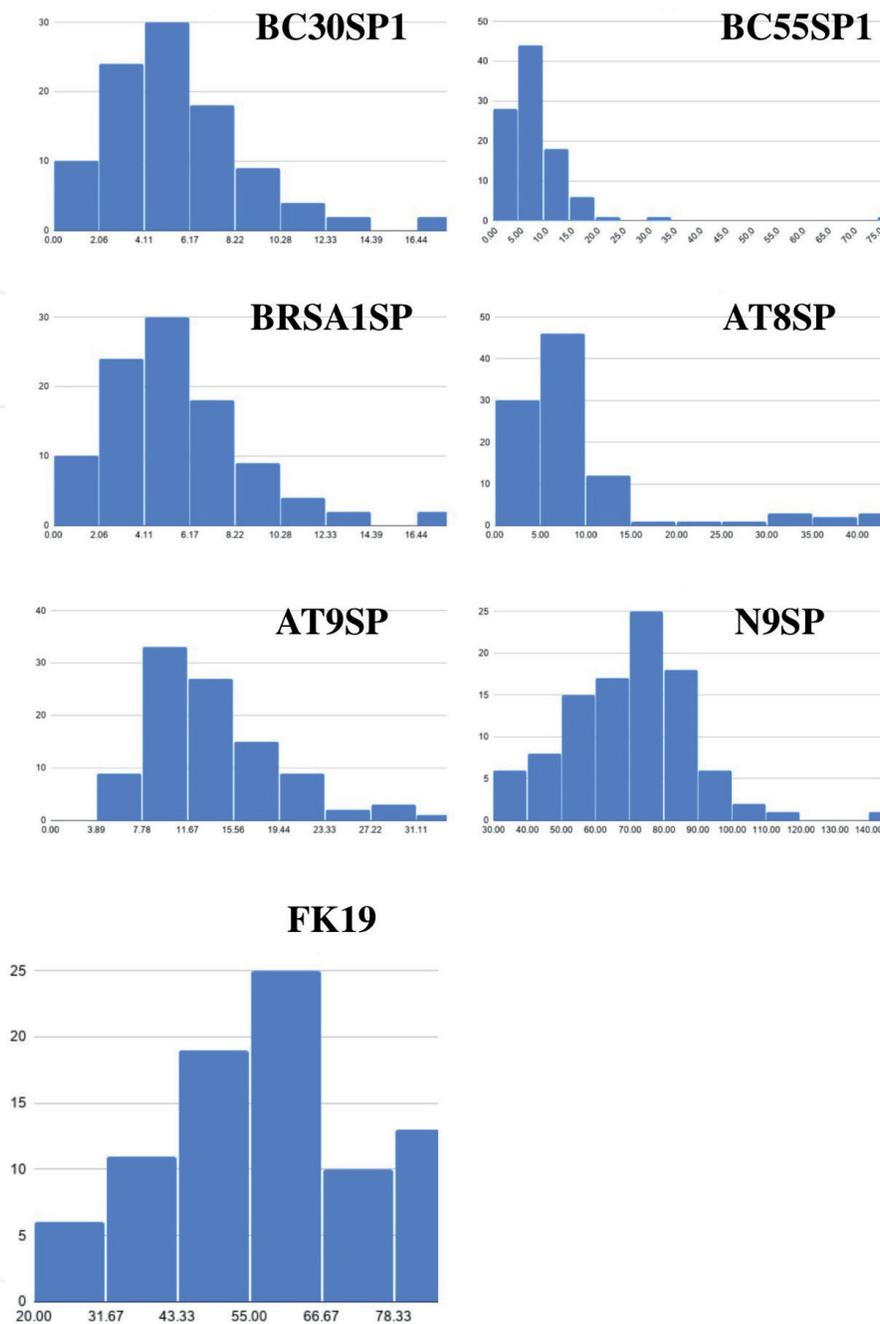


Figure 6.
 Histograms of fluorescence intensity distribution of H3K27me3.

3.3 Fluorescence intensity of SIRT1 and SIRT6

The average fluorescence intensity levels of SIRT1 (**Figure 8**) in patients with breast cancer did not significantly differ from the levels in the lines of healthy donors, carriers of the mutations in the *BRCA1* gene, and the mother of one patient with AT. However, the average SIRT1 fluorescence intensity in the line of the second mother of the patient with AT was significantly 13 times higher than in the lines of patients with breast cancer.

The average fluorescence intensity levels of SIRT6 were also the lowest in the lines of patients with breast cancer and patients with a mutation in the *BRCA1*

H3K9me3

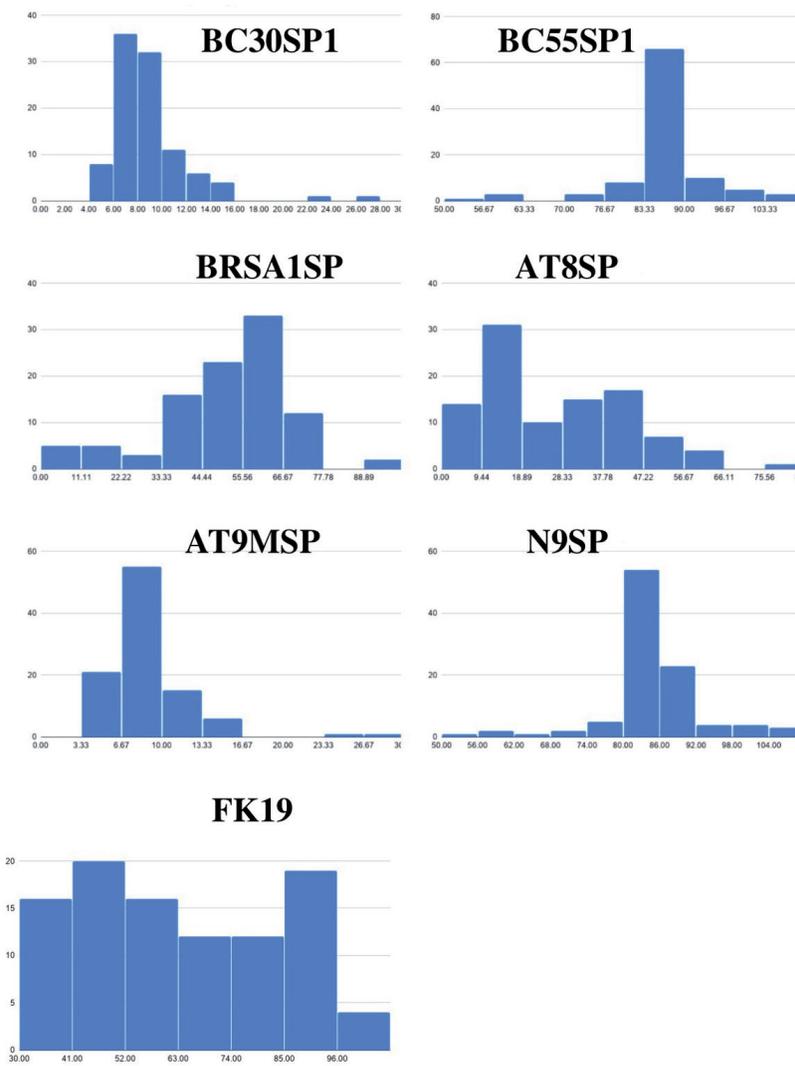


Figure 7.
Histograms of fluorescence intensity distribution of H₃K₉me₃.

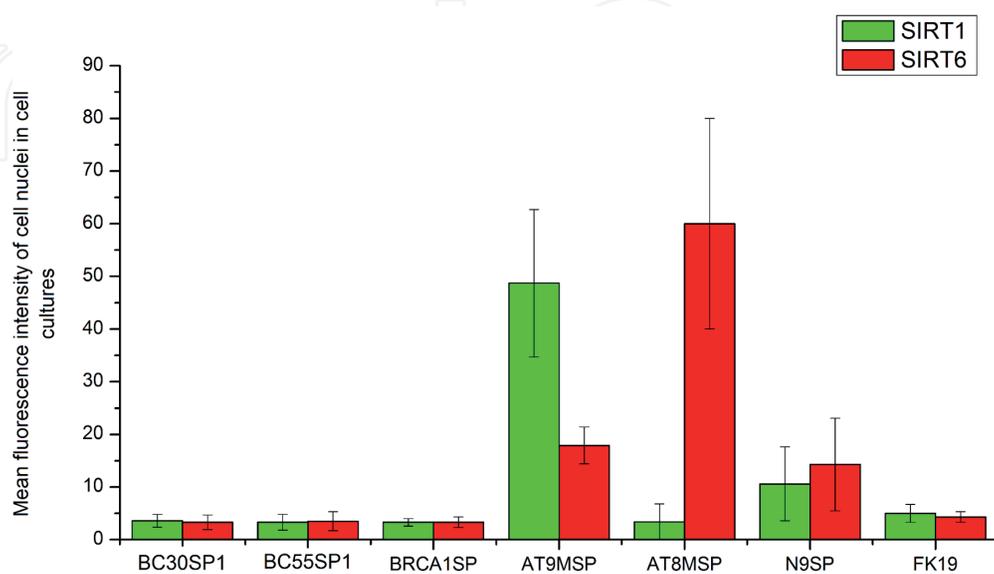


Figure 8.
Average fluorescence intensity of SIRT1 and SIRT6 in the nuclei of dermal fibroblasts of the studied lines.

SIRT6

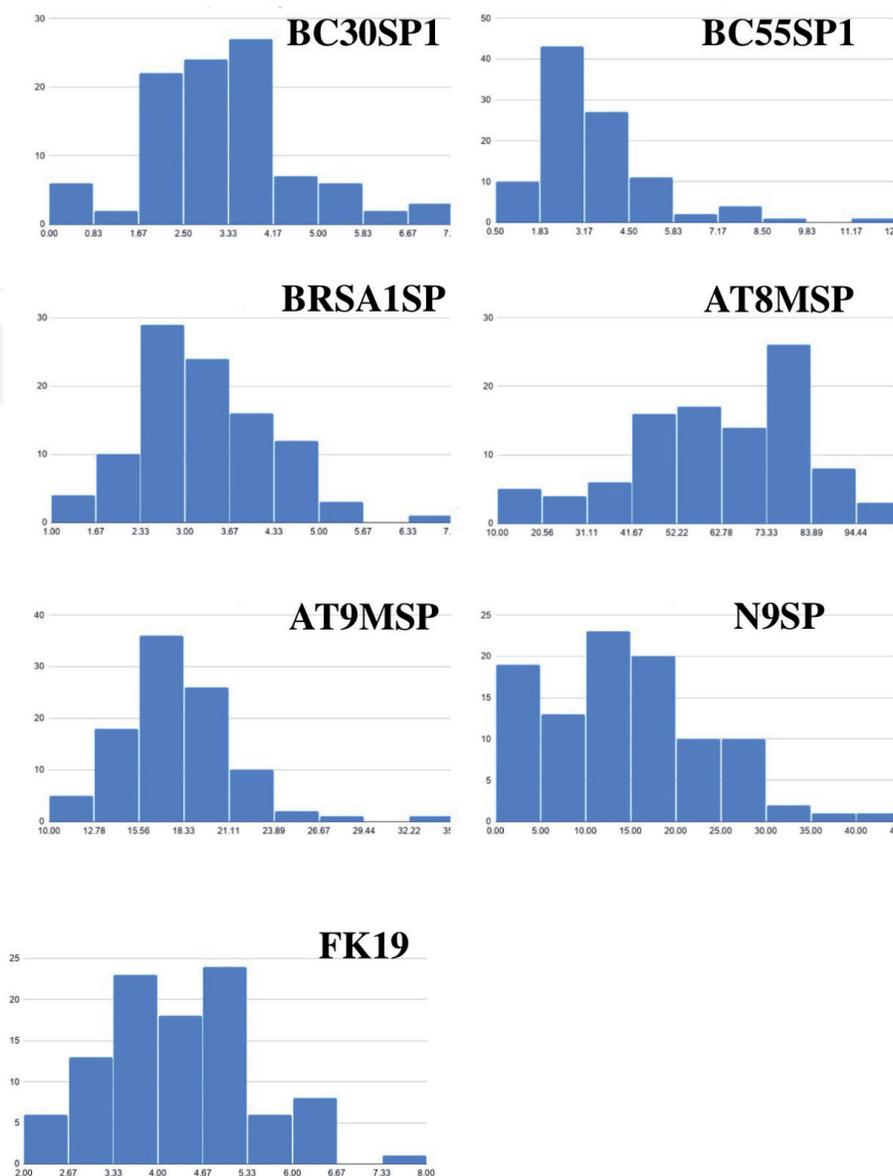


Figure 9.
 Histograms of fluorescence intensity distribution of SIRT6.

gene. Significantly, the levels of the average fluorescence intensity in the lines of patients with breast cancer did not differ from the levels of healthy donors, while they were significantly lower by 5 and 18 times the values in the lines of mothers of patients with AT.

Analysis of the histograms of the distribution of the fluorescence intensities of SIRT6 (Figure 9) and SIRT1 (Figure 10) in cell lines revealed two subpopulations with different distributions in the AT8MSP line according to the SIRT6 fluorescence intensity and in the AT9MSP line according to the SIRT1 fluorescence intensity. In the N9SP and FK19 cell lines, several significant peaks in SIRT6 fluorescence intensity were observed.

When overlapping histograms of SIRT6 and SIRT1 fluorescence intensity distributions in different cell lines with the same bin size, it is possible to observe that the widest range of SIRT6 fluorescence intensity is in the AT8MSP cell line and that of SIRT1 fluorescence intensity is in the AT9MSP cell line (Figure 11).

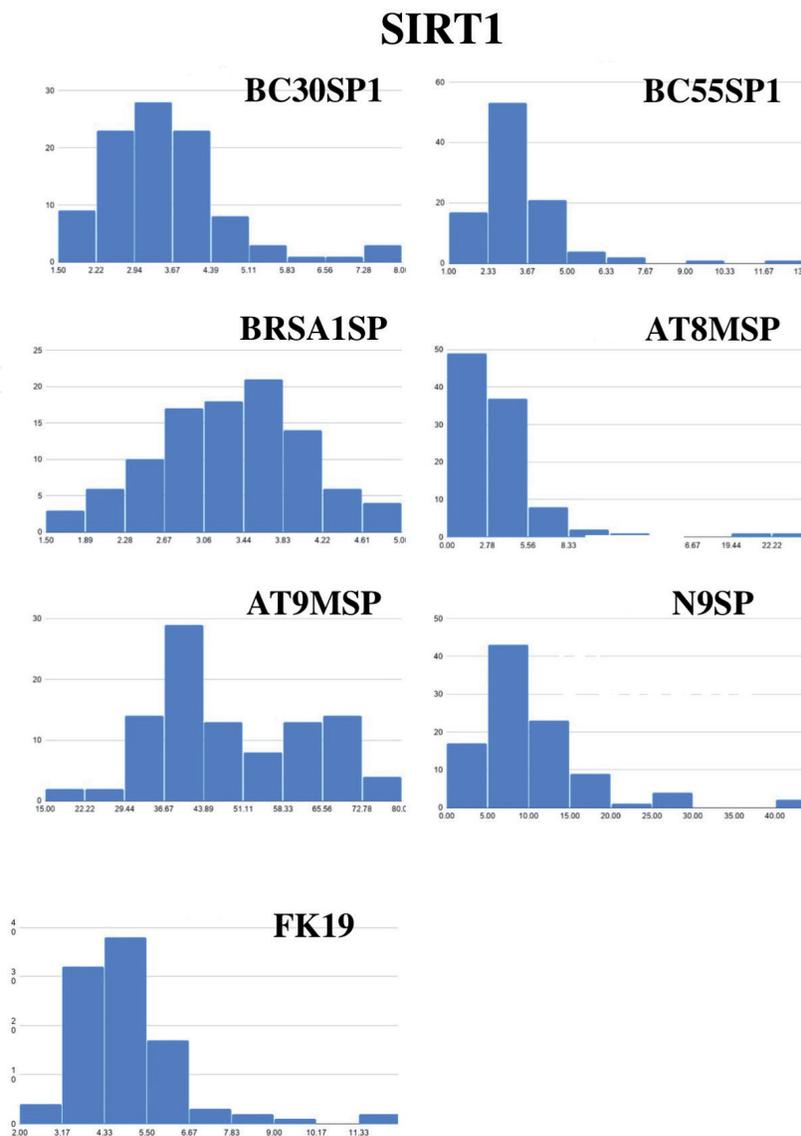


Figure 10.
Histograms of fluorescence intensity distribution of SIRT1.

4. Conclusions

Modulation of chromatin structure through modification of histones is the main epigenetic mechanism and regulator of gene expression. However, the contribution of chromatin properties to the heterogeneity and evolution of tumors remains unknown. Epigenetic labels are critical regulators of chromatin and gene activity. Their role in normal physiology and pathological conditions, including the development of cancer, is still unclear.

High expression of immune control points in the tumor microenvironment plays an important role in suppressing antitumor immunity, which is associated with poor prognosis and cancer progression. Major epigenetic modifications of both DNA and histone may be involved in the activation of immune control points in cancer.

Of course, for a more accurate study of epigenetic differences and features in breast cancer, a wider sample of the studied groups is needed. However, our results allow us to draw some preliminary conclusions in this matter. The results of our work showed significant differences in the lines of patients with breast cancer from the values of healthy donors only in terms of the average fluorescence intensity of H3K9me3 and H3K27me3. Contrary to published data, the average value of

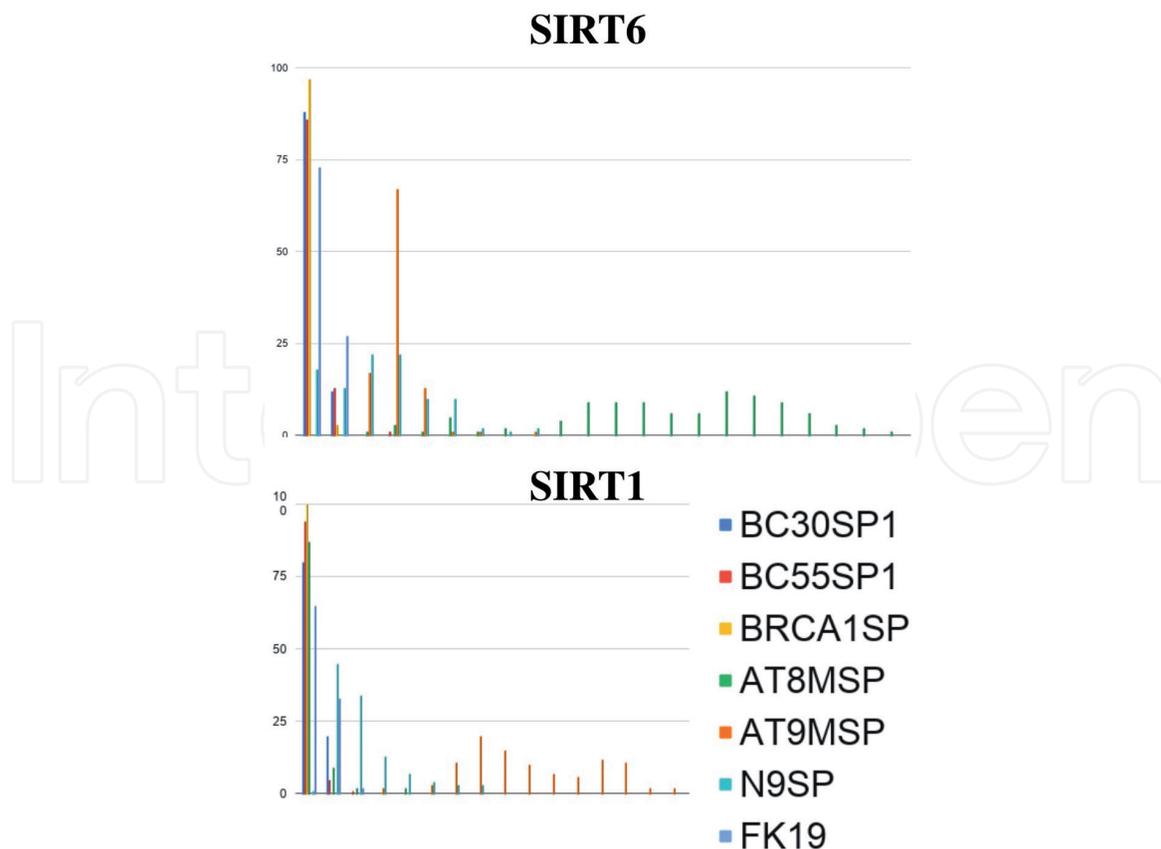


Figure 11.
Overlapping histograms of the distribution of fluorescence intensities of SIRT1 and SIRT6 in the studied lines with bin size of 5 RFU.

H3K27me3 fluorescence intensity in the cells of patients with breast cancer was significantly lower than in cells of healthy donors. Most likely, this is due to the fact that the study was not carried out in tumor cells.

Significant differences between the lines of patients with breast cancer were observed only in the values of the average intensity of HP1 γ . The average HP1 γ fluorescence intensity in a patient with breast cancer of 30 years was the largest of all the studied lines and in a patient with breast cancer of 55 years the lowest. The HP1 γ fluorescence intensity in the line of 30 years old breast cancer patient was 8.25 times higher than in the line of 55 years old patient. Our results suggest that heterochromatin protein is a potential marker for breast cancer.

Thus, our results confirmed data on changes in the amount of H3K9me3 and H3K27me3 proteins in breast cancer.

An analysis of histograms of fluorescence intensity distribution of HP1 γ , H3K27me3, H3K9me3, SIRT1, and SIRT6 allowed us to see how differently the fluorescence intensities are distributed in the cell lines and also allowed us to distinguish two cell lines with two subpopulations of the fluorescence intensity of HP1 γ (BC55SP1 and BRSASP1). However, for other cell markers, two subpopulations in this line were not detected. A second minor peak in HP1 γ fluorescence intensity was detected in the BC30SP1 cell line, but the distribution pattern was different from the distribution in the BC55SP1 line.

Interestingly, a pattern of fluorescence intensity distribution differed in different lines. But in most cases, it was similar within the same cell line in terms of fluorescence intensities of the HP1 γ , H3K27me3, H3K9me3, SIRT1, and SIRT6 proteins.

Usage of average fluorescence intensity value as a single characteristic is not sufficient to properly compare different cell lines, because it masks significant variations in fluorescence intensity distribution. Overlapping histograms of fluorescence

intensity distribution make it possible to assess variability of a given protein in a population.

The broadest range of fluorescence intensity values was demonstrated by the BRCA1SP (HP1 γ fluorescence intensity), AT8SP (SIRT6 fluorescence intensity), and AT9SP (SIRT1 fluorescence intensity) lines.

But on average, across all cell lines, the broadest range of fluorescence intensity values was shown by histones H3K27me3 and H3K9me3. The data obtained suggest that these proteins are more “sensitive” to the state of the cell during normal life cycle and during pathological changes.

Thus, our results confirm changes in the amount of H3K9me3 and H3K27me3 proteins in breast cancer.

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