Implications of miR-9 gene methylation in the appearance and development of breast cancer in early stages

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In summary presents the main results from their research. Numbering of figures, graphs and tables is equivalent to that of the sentence.

INTRODUCTION

Breast cancer is one of the most common malignancy in women worldwide, appearing annually with more than one million new cases.

Epigenetics is the study of inherited changes in gene expression or on cellular phenotype caused by mechanisms other than changes underlying DNA sequence – from were the name *epi-* (gr. over, above) -genetic and refers to relevant changes functionality in genome that do not involve changes on the nucleotide sequence. Examples of such changes are DNA *methylation* and histone *deacetylation*, both of them serving to suppress gene expression without altering the sequence of silent genes.

Epigenetic disorders have been studied extensively, leading to the idea that DNA methylation and microRNAs could play an important role in the development and progression of mammary neoplasia.

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I. GENERAL CONSIDERATIONS

CHAPTER 1. ANATOMY AND PHYSIOLOGY OF THE MAMMARY GLAND

Mammary glands are transformed sweat glands, modified, that develops from a thickening of the ectoderm. Are glands of ectodermal origin.

CHAPTER 2. CANCER

Cancer is represented by an abnormal increase of the cells, caused by the multiple changes in the gene expression, leading to an imbalance of proliferation and cell death and eventually evolve into a population of cells that can invade tissues, spreading to different regions of the organism, leading to a significant morbidity and, in untreated cases, death of the host (Raymond W. Ruddon, 2006, J. Gertner, 2004).
CHAPTER 3. PROCESS OF METASTASIS

The process of metastasis unequivocally marks the difference between malignant and benign tumors.

The metastasis is the transport of live cells from a primary pathological process and submitting them to other tissues and organs that generate lesions similar or identical to those of origin, called metastases.

CHAPTER 4. EPGENETICS

The one who stated the theory of epigenesis was Aristotle bet on book “On the Generation of Animals”.

4.1. DNA methylation

In vertebrates cells, cytosine methylation provides a powerful mechanism by which gene expression is transmitted to progeny cells.

- DNA methylation principle

Study of DNA methylation is based on molecular biology methods equipped with a high sensitivity and specificity. The evaluation, regardin the gneomic DNA, may be achieved on fresh material, or on preserved material. Sodium bisulphite converts selective residues of cytosine to uracil, leaving unchanged the cytosines related to carbon in position 5 (5mC) (Figure 13).

4.2. DNA methylation and microRNAs

DNA methylation refers to the covalent post-replicative addition of a methyl group (-CH_3) at the carbon in position 5 of cytosine.

4.3. MicroRNAs Biogenesis

MicroRNAs biogenesis in the human cell is a complex process formed by many steps. This process begins in the nucleus, and it is continued in the cytoplasm.

II. PERSONAL CONTRIBUTIONS

CHAPTER 5. MATERIALS AND METHODS

5.1. Patients and samples

Biological material: 31 samples of tumoral breast tissue, six samples of healthy breast tissue, 15 samples of tumoral lymph nodes, five samples of normal axillary lymph nodes, preserved at -80°C, 66 samples from patients with breast cancer which were undergone to a surgery in Breast Unit, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo (FG), Italia (20-fixed with formalin and FFPE paraffin-embedded, 46-preserved at -80°C).

5.2. Methods used in the research of tumor types

5.2.1. Histochemical staining

H_2O – 10 seconds
5.2.2. Histopathological method
For a detailed analysis of histological components, their processing was performed in paraffin.

5.2.3. Immunohistochemistry
In a later stage, 46 of the 97 cases of breast cancer were processed for immunohistochemistry, cases that had clinical and morphological data.

5.2.4. DNA extraction from frozen tissue
DNA extraction from fresh frozen tissue, which was subjected to digestion with proteinase K (Pk), was performed according to the protocol (Parrella P et al., 2001).

5.2.5. DNA extraction from paraffin-embedded FFPE tissue
The 20 samples of paraffin-embedded FFPE tissue were used for the DNA extraction, that was performed according to the protocol (see page 123-Thesis).

5.2.6. Sodium bisulfite conversion
The isolated DNA from normal and tumoral tissue was subjected to bisulfite treatment and purification according to the protocol EpiTect Bisulfite kit (Quiagen Sci, MD USA).

5.2.7. Primers design
miR-200c: Forward - 5'-CGTGGTGTTTGCCTAGG-3'
Reverse - 5'-CGACCAGCGACAACACCAGG-3', 188bp fragment size.

miR-9: Forward - 5'-CGCAGCTGCTGTAATG-3'
Reverse - 5'-CGCCGGAACCTTTTCTACCC-3', 130bp fragment size.

β-actin (ACTB): Forward - 5'-TGGTGATGAGGGAGGTATAGGT-3'
Reverse - 5'-AACCAATAAACCTACTTCCTACCA3' by Invitrogen.

CHAPTER 6. RESULTS AND DISCUSSION

6.1. STUDY OF MIR-200C AND MIR-9 GENES METHYLATION ON PATIENTS WITH BREAST CANCER
The aim of this study is to determine the implications of miR-200c and miR-9 methylation genes in the appearance and development of early mammary neoplasia, by MS-PCR (MSP).

6.1.1. MiR-200c gene and its implications in cancer
In molecular biology, miR-200 is a short molecule of RNA (microRNA). MiR-200 family contains miR-200a, miR-200b, miR-200c (located on 12 chromosome) (Fig 19), miR-141 and miR-429.
6.1.2. Frequency of miR-200c promoter region gene methylation in patients with mammary neoplasia

![Graph showing frequency of miR-200c promoter region gene methylation in normal and tumoral breast tissue.]

\[ P_{val} = 0.2 \]

Charter 1. Frequency of miR-200c promoter region gene methylation in normal and tumoral breast tissue

![Images of gel electrophoresis showing miR-200c promoter region gene methylation in normal and tumoral breast tissue.]

Figure 19. MiR-200c-141 cluster located on chromosome 12

Figure 21. MiR-200c promoter region gene methylation in normal and tumoral breast tissue (down); amplification with β-actin (up). Legend figure 21. B – blank, 86, 81, 75, 71- mammary tumors, 4, 2, 3r (right breast), 3l (left breast), 1r (right breast), 1l (left breast).

Figure 22. MiR-200c promoter region gene methylation in tumoral breast tissue. Legend figure 22. B – blank, M – metilated, U – unmetilated

6.1.3. MiR-9 gene and its implications in cancer

MiR-9 precursor (counterpart of miR-79), located on chromosome 1, in 1q22 position in humans (Figure 23), in a short gene ncRNA (non-coding RNA) involved in gene regulation. MiR-9 is processed by 5' end of its precursor, and miR-79 by 3' end.

![Diagram of miR-9 gene location on chromosome 1 (1q22).]

Figure 23. miR-9 gene location on chromosome 1 (1q22)
6.1.4. Frequency of miR-9 promoter region gene methylation in patients with mammary neoplasia

6.1.5. Conclusions

- Possible role of miR-9 methylation in appearance and development of early breast cancer.
- miR-200c is not a target in breast cancer prognostic.
- miR-9 is shown to be methylated in 64% in tumoral tissue, and in none of the normal tissue, which indicate the possibility of this gene to become a marker in breast cancer prognostic.

6.2. STUDY OF MIR-9 GENE METHYLATION ON AXILLARY LYMPH NODES ON PATIENTS WITH BREAST CANCER

The aim of this study is to determine the implications of miR-9 methylation genes on lymph nodes in the appearance and development of early mammary neoplasia, by MS-PCR (MSP).

6.2.1. Correlation between miR-9 promoter gene region methylation in lymph nodes with clinical data of the patients with breast cancer
6.2.2 Conclusions

- miR-9 gene methylation has an important role in appereance and development of breast cancer.
- miR-9 gene methylation has a very important role in tumoral invasion, representing a target for breast cancer prognostic.

6.3. STUDY OF MIR-9 GENE METHYLATION ON ON PATIENTS WITH BREAST CANCER, WITH IMPLICATIONS IN THE DEVELOPMENT METASASES

The aim of the study is to determine the methylation process involving miR-9 gene, on a well defined group of patients, which include 66 tumor samples, from which 20 samples are FFPE tissue and 46 samples are frozen tissue, and six normal breast tissue (healthy).

6.3.1. Determination of different histological types of mammary neoplasia

Staining with hematoxynil and eosin is the most common histological staining method.
6.3.2. Correlation between miR-9 promoter gene region methylation with clinical data of the patients with breast cancer

**Figure 30.** a. Normal breast tissue; b. ADH (atypical ductal hyperplasia); c. DCIS (ductal carcinoma “in situ”); d. IDC (invasive ductal carcinoma) – 20X

**Charter 8.** Frequency of miR-9 methylation in normal and tumoral breast tissue

**Charter 9.** Frequency of miR-9 methylation in normal breast tissue and margin of safety

**Charter 10.** Frequency of miR-9 methylation in margin of safety and tumoral breast tissue

**Figure 33.** MiR-9 promoter region gene methylation in diverse samples of breast cancer. Legend figure 33. Mk (molecular weight marker), B – blank, C+ (positive control), C- (negative control), I (IDC - invasive ductal carcinoma), S (normal tissue), D (DCIS - ductal carcinoma in situ), A (ADH – atypical ductal hyperplasia).
Charter 13. Distribution of distant metastases

6.3.3. MicroRNAs in human genome – Many major cellular functions like development, differentiation, growth and metabolism are regulated by the microRNAs.

6.3.4. MicroRNAs in other genomes – Cellular functions of miRNAs seem to vary in eukaryotes, including the regulation of leaves and flowers development in plants (Aukerman MJ, 2003), and modulate hematopoietic cell differentiation in mammals (Chen CZ, 2004).
6.3.5. MicroRNAs in human diseases - It has been shown that microRNAs play a major role in a wide range of developmental processes including metabolism, cell proliferation, apoptosis, development cycle, and neuronal cell fate (JS Mattick, 2005).

6.3.6. MicroRNAs in cardiovascular diseases - Studies have shown that 3 miRNAs (miR-1, miR-133 and miR-208) are overexpressed in the heart (Baskerville S, 2005, Lagos-Quintana M, 2002) and are important regulators of heart development and in differentiating of myocytes (Zhao Y, 2005, Chen JFet al., 2006, Van Rooij E, 2007, Zhao Y et al., 2007).

6.3.7. MicroRNAs in inflammatory diseases - Inflammation is an essential component of the host defense system and a response to major infections and injury, which is believed to contribute to many chronic and acute diseases (Ross R., 1999, Silvestre JS, 2008).


6.3.9. MicroRNAs in cancer - It is now well known that miRNAs upregulation or downregulation occur in different human cancers (Moslemi Naeini M, 2009).

6.4. Association between ER, PR and hsa-miR-9-1 promoter gene region methylation frequency - Methylation of studied area has a high frequency in breast cancer ER + and PR +.

6.5. Association between HER2 and hsa-miR-9-1 promoter gene region methylation frequency - MiR-9 promoter region gene methylation shows a frequency higher in luminal cancers and is has a lower frequency in bazal HER2-dependent cancers.

6.6. The role of miR-9 gene in appearance and development of metastases - Recent data suggest that aberrant expression of miR-9 may be involved in metastasis formation (Amaia Lujambioa, George A. Calin, 2008).

6.7. Analysis of results from a therapeutic standpoint - Patients were subjected to various treatments, such as chemotherapy, radiotherapy, hormone therapy and immunotherapy.

6.8. Analysis of survival rate - All patients with distant metastases died. Cox univariate and multivariate regression analysis showed no significant association between miR-9 methylation and survival (OS), progression-free survival (PFS) and metastasis free survival (MFS).

6.9. Conclusions

- Methylation is not present in normal breast tissue obtained by reductive mammoplasty, only in breast tumor tissue.
- Also, the results show that hsa-miR-9-1 gene methylation is more common in tumor than in healthy breast tissue.
- MiR-9 gene methylation was more pronounced in samples obtained from patients with bone metastasis than in those obtained from patients with visceral metastases.
- Methylation frequency was higher in samples obtained from patients who developed bone metastasis as first metastatic site.

**GENERAL CONCLUSIONS**

- About miR-200c promoter region gene methylation, there are significant differences, both in healthy breast tissue and in breast tumor tissue (pval = 0.2).
- MiR-9 gene methylation is frequent in breast tumor tissue than in healthy breast tissue, were is absent (pval = 0.004).
- Methylation it is more common in lymph nodes tumor versus normal breast tissue (pval = 0.01).
- Frequency of hsa-mir-9 promoter region gene methylation is higher in tumoral lymph nodes compared to the healthy lymph nodes, in which methylation was absent (pval = 0.003).
- No significant differences methylation in breast tumor tissue and lymph node tumors (P = NS - χ² test), methylation status was equally frequent in both types of tissue.
- According molecular data, that showed that the promoter region of miR-9 gene is methylated in 32 of the cases of breast cancer, correlated with clinical data on ER and PR, we can see that methylation of studied area has a high frequency in breast cancer ER + and PR +.
- The results indicate that miR-9 promoter region gene methylation is more common in cases of DCIS compared with ADH.
- MiR-9 gene methylation was more pronounced in samples obtained from patients with bone metastasis than in those obtained from patients with visceral metastases.
Methylation frequency was higher in samples obtained from patients who developed bone metastasis as first metastatic site.

Statistical test results showed no significant association between miR-9 gene methylation and survival (OS), progression-free survival (PFS) and metastasis free survival (MFS).

Also, miR-9-1 gene methylation increases the possibility of adjusting its expression by epigenetic mechanisms, which could have an important role in the mechanisms of formation of metastases in various organs.

In conclusion, the results indicate that miR-9-1 gene methylation is a common event in breast cancer.

BIBLIOGRAPHY


2. **Florescu Anca et al.**, FREQUENCY OF HSA-MIR-9-1 METHYLATION ON AXILLARY LYMPH NODES ON PATIENTS WITH BREAST CANCER, 2012.


7. Park SM et al., "The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2", 2008.


PERSONAL PAPERS


3. **Florescu Anca** et al., “miR-9-1 aberrant methylation is a frequent event in breast cancer and is associated with bone metastases”, în recenzie la BMC Cancer (ISI, IF 3,15).

