

Evolutionary Hypothesis in Cell Cycle of Breast Cancer Patients: Mosaic Phases in Single Cancer Cells

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Abstract: *Introduction:* Cell cycle shapes the initiation, progression and therapeutic approaches of neoplasms. An uncontrolled cell proliferation and growth are the key characteristics of either malignant or benign tumors. The programmed check points control the transition of phases through the related barriers. Therefore, balancing the carcinogenic processes may inhibit progression and facilitate a targeted-base therapy.

Methods: The present study is performed in interphase. Detection of the *Mosaic Phases (MPs)* by Fluorescence In Situ Hybridization was confirmed by assaying the protein expression (PE) including immunofluorescence and flow cytometry.

Results: The novel hypothesis reflects the presence of dual and/or multi-phases, as minor clones in single cells of breast cancer (BC) patients. This finding led to initiate a model with applicable ratio values and different *MPs* including G1/S, S/G2 and G1/S/G2, accompanied by normal phases (G1, S, G2). The remarkable harmonic behaviors between signal copy numbers and the corresponding PE, dual- and triple- co-expression between different cyclins combination including E/B1 and D1/E/B1 and the other involved proteins were observed. The ratio of gain to normal signals appeared to be a good prognosis for chromosome 1, but better survival was significantly obtained for this ratio in chromosome 3

Conclusion: The *predisposing-diagnostic-predictive-prognostic-preventive* panels may lead to innovate the CDKs inhibitor-based therapy by considering the *MPs* Model; and may also be considered for clinical classification, in BC and other cancers.

Keywords: Cell cycle, Mosaic Phases, evolutionary hypothesis, breast cancer, FISH, Protein expression.

INTRODUCTION

There is no available report on the *Mosaic Phases through the cell cycle*. An uncontrolled cell proliferation and growth are the key characteristics of neoplasms. The programmed check points control the transition of specific phases through the related barriers [1]. Besides, the proliferation, growth, or apoptosis; cyclin dependent kinases, cyclins and retinoblastoma (Rb1) gene are the fundamental requirements for a successful G1/S transition [2, 3]. CCND1 polymorphism rs614367 as an intragenic SNPs, affects the regulation and expression of CCND1 gene in breast cancer (BC) [4]. In fact, the functional capacity of these SNPs (Single nucleotide polymorphisms) is important to be considered.

The key events through the cell cycle is summarized [5 -7]:

1. At early G1, the complex of cyclin D/CDK-4 or 6 is responsible for the first phosphorylation of pRb in G1.
2. Cyclin E binds and activates CDK2, to phosphorylate pRb in late G1.

3. In S-phase, the complex of cyclin A/CDK2 leads to further functions.
4. Cyclin B1/CDK1 complex facilitate transition through G2-M phases.
5. Evolution and heterogeneity are detectable in metaphase or in interphase by classical and molecular cytogenetic investigation [8].

The aim of present study was to explore the functional targets involved in cell cycle which led to unmask the novel predictive/diagnostic/prognostic/preventive, as a quadrant –formulated-periodic profile based on the combined phases of the cell cycle-architecture in the single cells. The Complex/*Mosaic Phases* (CMP) may pave the way to innovate the new combined strategic therapy. However, cell cycle is capable to shape the initiation, progression and therapeutic approaches of neoplasms.

MATERIALS AND METHODS

Based on the diverse resting period of differentiated- and dividing-cells, most of the biochemical activity of the nucleus occur during interphase [9]. A chromosome is made of one chromatid in G1-, fragmented form in S- and two chromatids in G2- phase [10]. Detection of the *MPs* by Fluorescence In Situ Hybridization (FISH) was confirmed by immunofluorescence (IF); and flow cytometry (FC) [11].

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Patients and Materials

The mean age of 60 female patients with BC was 48.4 ± 11.8 years (range: 26-78 years).

Pathological classification included Invasive ductal carcinoma (n=45), atypical medullary carcinoma (n=3); Invasive lobular carcinoma (3) and others (n=10).

Based on the involvement of chromosomes 1, 3 and 8 in the patients' karyotype, 38 out of 60 have been selected for this project. Cell cycle phases were determined by the results of FISH assay, with application of three centromere probes including chromosome 1 (Green), 3 (orange) and 8 (Oncor, Germany and Cytocell, UK). Two probes including CCND1 (green) and MYC (orange) (Metasystems, Germany) were also tested.

FISH Technique

Fresh tumors were fixed by Methanol, spotted on the slide and incubated in 2x SSC (Saline Sodium Citrate). FISH probes were added, cover-slipped, incubated at 75°C for 7 minutes, and hybridized at 37°C overnight. Then slides were treated with 0.4 x SSC for 5 min, were incubated in 2 x SSC, 0.05% Tween 20 for 3 min at room temperature, rinsed briefly in distilled water and were air-dried. DAPI/anti-fade (4', 6-diamidino-2-phenylindole) was added and concealed with cover slip and incubated in the dark for 10 minutes before scanning with a Fluorescence microscope (Leica, Germany). Scoring was performed by enumeration of signals in 100-500 nuclei of each sample with 40x and 1000x magnification.

Protein Expression

The mode of PE was, assayed by Flow cytometry [11]. Immunofluorescence was performed for Cyclin D1 (DCS-11 Biosource, Belgium), Cyclin E (Zymed, USA), Cyclin B1 (7A9, Vision Biosystem/Novacastra), CDK1 (ABIN598555, ABCAM, Germany), Cyclin A (clone:6E6; Novacastra, Newcastle-Upon-Tyne, UK), CDC25A (DCS121, Sigma, USA), P21 (SX118, Dako, Denmark), PCNA (PC10, Dako, Denmark), Ki67 (Dako, Denmark), CD44, and CD24 (HP2/9; SN3, GENTAUR, Brussels).

Characterization of Cell Cycle Phases

The CMP is characterized as Multi-phases cyclic pattern, multi-programmed-potential, and the multi-targeted therapeutics candidate.

Ratio values was calculated based on the frequency of *MPs* between chromosomes- 1/1, 3/3 and 1/3 by considering the variables A-L, between different complexes of dual and triangle cell cycle phases. Distribution of the PE was explored according to the expression intensity including lack, moderate and high levels.

Statistical Analysis (SA)

The status of *MPs* by considering the clinical-cellular characteristics was analyzed by the chi-square test. Overall survival (OS) was estimated using the Kaplan Meier product limit method. SA was performed using the IBM statistic 22. The two-tailed statistical tests, and P-value <0.05 was significant.

The survival period was estimated based on the initial date of diagnosis per month and followed up to the deceased date. The histo-pathological classification was provided by the surgeons. Based on the survival through diverse *MPs*, the predictive value was provided as hazard ratio and corresponding 95% confidence intervals by application of the Cox proportional-hazard models. The Wald Z-statistic P value was performed for all the variants. To adjust the multi-variant analyzes, the cox regression model was used for different clinical-, family history classification, different types and combined therapies, metastatic- and Er status.

RESULTS

Distribution of diverse signal-based characteristics of *MPs* through the cell cycle were statistically compared with the patients'- age, family history of cancer, and clinical-histo-pathological characteristics. Patients without FH had higher frequency of *MPs* in chromosome 1 (P=0.013). The ratio of total signals was significantly high in patients with positive FH (P=0.030) (Table 1a).

The significant differences were found neither for *MPs* nor patients' age, nor between deceased and alive patients. But, patients with Er+ had higher ratio of signals for chromosome 3 than with Er- (P=0.018). The ratio of signal's loss to normal for chromosome 3 was significantly high in patients with Er+ (P=0.047) (Table 1b).

No significant differences were found between deceased and alive patients who received single or triple therapies. But, the cumulative signal gain in chromosome 1 was significantly higher in alive- than in deceased patients who have been treated with duplicated protocols (P=0.05) (Table 1c).

Table 1: Descriptive and Significant Values of Signal-Based Mosaic Phases between Patients with Different Clinical Characteristics, and Ratios

	Frequency	Mean	St. Deviation	Std. Error	P-value
1a					
†1.SumMPCh1					
0	19	9.4737	11.52470	2.64395	.013
1	21	2.4286	4.33095	.94509	
†2. RSig/MPs					
0	15	14.5905	16.57480	4.27959	.030
1	15	43.4403	46.05284	11.89079	
1b					
‡1.SigMP/NCh3					
0	10	8.4142	7.60980	2.40643	0.018
1	5	34.4086	29.52600	13.20443	
‡2.Sig.loss/NCh3					
0	12	1.5204	1.28826	37189	.047
1	11	3.9605	3.77061	1.13688	
‡3.Sig./TotMPs					
0	17	13.8200	13.13072	3.18467	.013
1	12	47.3352	50.08012	14.45689	
1c					
¶ CumSigGain Ch1					
A	16	23.5000	19.15550	4.7888	0.05
DC	4	30.000	5.35413	2.67705	
1d					
§1.RSigMPCh3					
0	14	12.7989	13.62601	3.64171	0.001
1	1	77.0000			
§2. RSigloss/Nch3					
0	21	2.0529	2.01397	43948	0.000
1	2	9.3500	4.03051	2.85000	
§3.RSigTot/MPs					
0	28	25.2554	28.86061	5.45414	.035
1	2	81.6563	106.55215	7534375	
1e					
††SUMTotSig:					
0	10	99.7000	37.33646	11.8068	0.036
1	2	166.5000	9.19239	6.50000	

Table 1 includes sections 1a-e.

0: Negative; 1: Positive; N: frequency; Ch: chromosome; MP: mosaic phases, R: ratio, Sig:signal; Tot: total.

1a. Significant values of signal-based mosaic phases between patients with- and without family history of cancer.

†1. Sum of Mosaic phases of chromosome1; †2. Ratio of signals/ MPs.

1b. Descriptive values for different characteristics of Signal-based mosaic phases and estrogen receptor expression.

‡1. Signal of mosaic phases/normal chromosome 3; ‡2.Signal loss/ normal chromosome 3; ‡3.Signals/Total of MPs.

1c. Descriptive values for different characteristics of signals and MP between deceased and alive Patients who receive duplicated therapies.

¶ CumSigGainCh1: cumulative of signal gain for chromosome 1; A: alive; DC: deceased.

1d. Descriptive and significant values for different characteristics of signals and mosaic phases, considering the presence of Medical complication.

§1.Ratio Signal of MPs/ normal chromosome 3; §2. Ratio of signal loss/normal chromosome 3; §3. Ratio of signal Total/MPs; 0: No medical complication (MC): 1: with MC.

1e. Descriptive values for characteristics of signals, mosaic phases and metastasis status of patients who have received one therapeutic protocol.

††: Sum of total signals; 0: lack of metastasis; 1: positive metastasis.

The patients with medical complications had higher ratio for signals of chromosome 3 (P=0.001); for ratio of signal loss to normal in chromosome 3 (P=0.000), and

for ratio of total signals (P=0.035) (Table 1d). The value of total signals between metastatic status of patients receiving one therapeutic protocol was

significantly higher in patients with metastasis ($P=0.036$) (Table 1e).

Significant differences were not found for pathological classification, the tumor laterality, size; and the status of auxiliary lymph node.

Survival is distributed amongst independent variables, so the *MPs* variables were categorized into the lower quantity and higher degree (Table 2). In patients who received only chemotherapy, the survival distribution between two groups is summarized (Table 2a). For chromosome 1, distributions of survival were increased across the two strata of loss to normal ratio of signals which were consistent significantly with chromosome 3 (17.0 ± 0.8 and 132.7 ± 10.1 , $P=0.05$). The loss of signals in chromosome 1/3 ratio remarkably decreased across the strata (145 ± 12 , 51 ± 27.7 , $P=0.246$). The survival of patients was high in first stratum of the total signals and *MPs* (Table 2a).

Distribution of survival across different variables of *MPs* and signals for patients who received chemo- and hormonal therapies were summarized (Table 2b). Signal gains in chromosomes 1 vs. 3 had significantly poor influence on the patients' survival (90.5 ± 38 and 29 ± 1 , $P=0.048$) (Table 2c).

Evolutionary Model of the CMP

Thirty combinations of different phases are provided through the evolutionary course (Figure 1). The most frequent involved *MPs*-complex included G1/S (81.70) and G1/G2 (42.43) for chromosomes 1 and 3; followed by 1,7; and 12,1 for S/G2 and G1/S/G2 respectively.

Ratio of chromosome 1/3 with either >1 or $=1$ frequency, lacking *MP*, is observed in 20 patients. Ratio of chromosomes 1/3, totally, lacking *MP*, is detected in two patients with 5G1/1s and 5G1/1S/2G2 combinations (Figure 2).

Dual functional results by FISH and PE in breast tumors (BTs) are provided (Figure 3).

Status of *MPs*, and PE of cyclin D1, E and B1 is diverse (Figure 4). The mode of PE for stem cell, cyclin E, B1 and Ki-67 are remarkable (Figure 5), the complex of cyclin B1 and CDK1 was also tested. PE of CD44 and CD24, cyclin- E, B1 and CDC25A is also presented (Figure 6).

DISCUSSION

According to the *MPs* hypothesis, the malignant cells may be characterized with partially or totally 'NON CYCLIC'. Cyclin D is regulated through induced proteolysis. Activation of Ras leads to transcriptional induction of cyclin D1 through a responsive mechanism of Ras [12].

Constitutive heterochromatin (CH) is located in C-band and often at centromere; its simple repeated DNA sequences, corresponds to the satellite DNAs; "Heterochromatic segments (HSs) tend to fuse in interphase"; and the HSs seem to be more "chromocentres or prochromosome" in interphase [13, 14]. It is stated that "The ignorance of the true role of heterochromatin has left the field open for a variety of hypothesis" [15]; and followed by "its important function in development and evolution" [16, 17]. However, '*MPs*

Table 2: Cumulative Distribution of Survival through Tertiles for Variables of Mosaic Phases of Chromosomes 1 and 3 according to the Clinical Parameters and Classifications

2a	Only Chem- OS (mo.)		
<i>MPs</i>	I	II	P-value
†. LossCh1/Loss Ch3	145±0	51±27.7	0.0246
††. Loss/norm Ch3	17	132.7±10	0.05
2b	Only Chem-OS	Chem+ horm-OS	
‡. LossCh1/loss Ch3	124±43	140	0.05
‡‡. Gain/norm Ch3	70±21	232±39	0.05
+. Sum total Sig Ch1,3	148	102±19.24	0.035
2c	Only Chem-OS	Horm +radio- OS	
§. Sig gain Ch 1/3	90.5±38	29±1	0.048

Table 2 includes sections a-c.

MP: mosaic phases; *Ch*: chromosome; *OS*: overall survival.

Treated with: 2a. Only chemotherapy; 2b. Chemotherapy; chemo- and hormone therapy; 2c. Chemo-, radio- and hormone- therapy.

†. Loss of Chromosome 1/3; ††. Loss/normal Ch 3; ‡. Loss of Ch 1/3; §. Signal gain of Ch 1/3; ‡‡. Gain/normal ch 3; +. Sum. total signals of Ch 1/3; §. Signal of Gain for Ch 1/3.

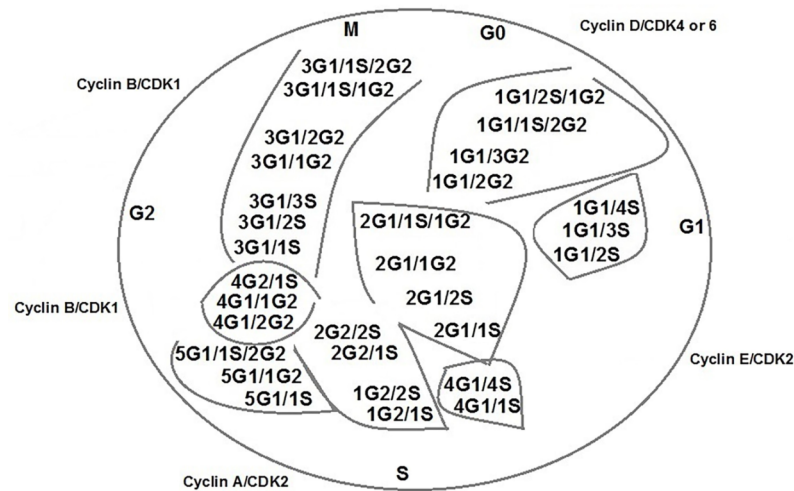


Figure 1: Distribution of mosaic phases through the cell cycle.

G: growth; S: synthesis; M: mitosis.

Left side Numbers of each phase: Frequency of signal copy numbers. Each line demonstrates combination of different phases.

CDP	Ch1	Ch3	R	CDP	Ch1	Ch3	R	CDP	Ch1	Ch3	R
1G1/2S	5	7	.71	3G1/1S	1	0	1/0	1G1/1S/2G2	1	1	1
1G1/3S	3	4	.75	3G1/2S	5	0	5/0	2G1/1S/1G2	1	0	1/0
2G1/1S	2	0	2/0	3G1/1G2	1	0	1/0	1G1/2S/1G2	1	0	1/0
1G1/2G2	2	0	2/0	3G1/2G2	2	0	2/0	3G1/1S/1G2	3	0	3/0
1G1/3G2	3	0	3/0	3G1/3S	0	1	0/1	3G1/1S/2G2	1	0	1/0
2G1/1G2	10	10	1	4G1/1S	6	5	1.2	5G1/1S/2G2	0	0	0
1G2/2S	1	0	1/0	4G1/4S	1	0	1/0				
1G1/3G2	2	0	2/0	4G1/1G2	1	0	1/0				
2G1/1S	42	20	2.1	4G1/2G2	1	0	1/0				
2G1/2S	4	1	4	4G2/1S	2	0	2/0				
2G2/2S	1	0	1/0	5G1/1S	0	0	0				
2G1/2G2	2	0	2/0	5G1/1G2	1	0	1/0				

Figure 2: Frequency and ratio indices for mosaic phases between chromosomes 1 and 3.

CDP: Combined different phases: G: growth; S: synthesis; M: mitosis.

Left side numbers of each phase: Frequency of signal copy numbers. Each line demonstrates combination of phases.

R: ratio between chromosomes 1/3 frequency; CDP: Combined different phases: G: growth; S: synthesis.

Each horizontal line demonstrates the frequency and Ratio of each combination of phases.

hypothesis' has unmasked the unexpected variations in cancer cells. Although it seems that CH has no effect on the phenotype, but, the polymorphic/predisposing factor in CH may play a role in cancer-phenotype.

In presence of 2 or more *MPs* in each malignant cell; the complete or partial proteolysis may not occur. Principally, regulated proteolysis is a periodic subject through the cell cycle and is possibly related to the process of forward direction in normal cell cycle

phases; and/or re-direction of the cells back to the previous stage, with an active protein. However, the reversibility of phosphorylation may be a reliable choice through the phosphatases' action to induce the backward directional process of cell cycle' pahases.

Mosaicism has most frequently occurred between G1/S for chromosome 1 and 3 (27.5% & 22.98% respectively); for G1/G2 (chromosome 1: 8.77% & chr 3: 15.11%); between S/G2 (chr 1: 7.14 % & chr 3: 4.16

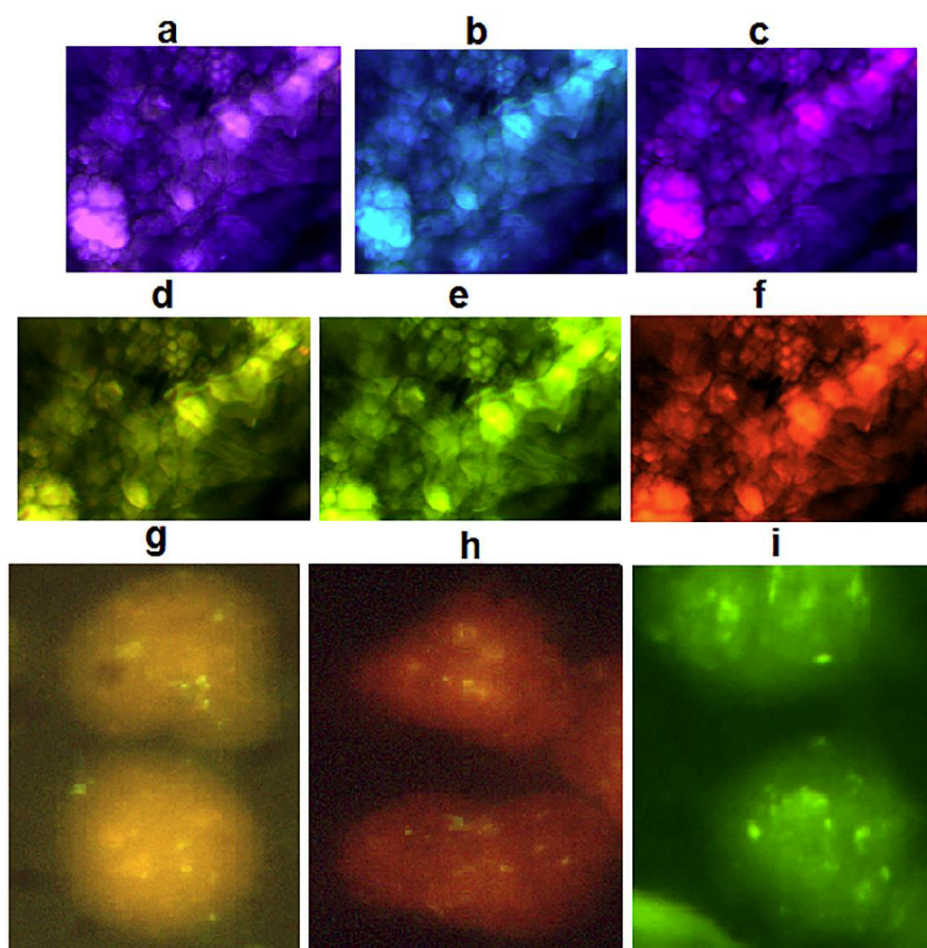


Figure 3: Status of signal copy number by FISH and protein expression by immunofluorescence in breast tumor.

Images **a-f**: PE. **g,h**: abnormal SCN as *MPs*: reflects G1, S, and G2 in each single cells. Single, dual and fragmented signals indicate G1; G2; S phases respectively. Merged images: **a**: dapi/cyclin D1 (Rpe); **b**: dapi/cyclin E (FITC); **c**: dapi/cyclin B1 (Pe-cy5); **d**: cyclinE/cyclin B1; **e**: Co-expression of cyclinD1/cyclin E; **f**: cyclinD1/cyclin B1; Bar: **a-f**: 10 μ m; magnification: **g- i**: 1000x.

%), and most frequently between G1/S/G2 in chromosome 1 (8.57%) and absolute lack for chromosome 3.

Although 5G1/1G2 complex is observed, combinations of 5G1/1S and 5G1/1S/ 2G2 are not detected. Conclusively, it seems that presence of the triple mode of *MPs* is limited to chromosome 1; and the frequency of cases in G1 within this category is limited to chromosome 1 (Figure 2). These findings vary in different cancers and chromosomes (unpublished data).

Initial, Dual and Triangle Model of *MPs*

The optional strategy for initiation and progression of such evolution may be due to the following progressive steps in which the categorization of events is based on the initial key step, i.e., cooperation of G1/S; G1/G2; and S/G2; G1/S/G2. However, G1 is the principal phase which evolves to the dual and/or

triangle combinations with 2 to 5 different phases (Figure 1). However, the initial step is accompanied by further evolution (multi- dual combinations of G1/S, S/G2, and G1/G2, or the multi- triangle/dual combinations (G1/S/G2, and/or G1/S or G1/G2). Therefore, the current check point barriers between different phases may be partially and functionally programmed (Figure 1). Furthermore, such course of evolution may have negative or positive impact on the clinical managements. Furthermore, the related proteins, either within the cell cycle or beyond, cooperate as the complementary developmental event (s) through the new cell cycle programming. The *MP* strategy may be, gradually, initiated at early stages through the individuals' life, at pre-natal and/or post-natal periods which could be prevented by implication of required standards of life style through different generations of the target pedigrees. However, cooperation between different phases is the secret of this evolution. By formulating the evolutionary puzzles,

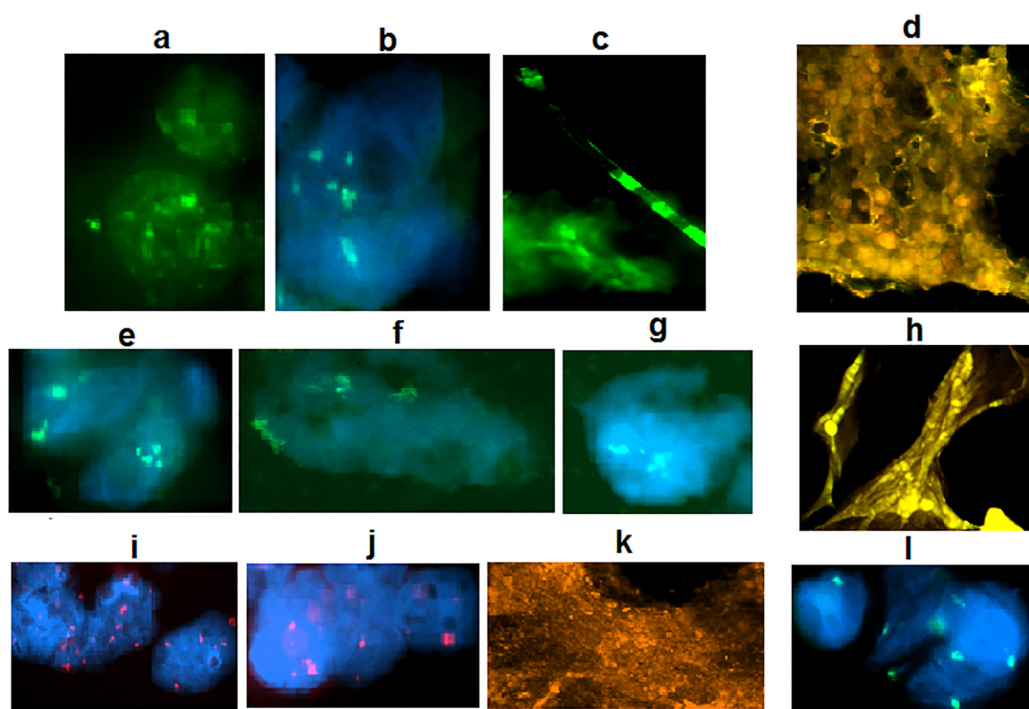


Figure 4: Status of signal copy number by FISH and protein expression by immunofluorescence in breast tumor.

a-c, e-g, i, j & l: SCN by FISH. Magnification: 1000x.

a. SCN of cyclin D1 with *MPs* (G1/S/G2); **b.** 1cen signals with *MPs* (4G1/1S); **c.** CTCs in vasculature & tumor, PE of cyclin E; **d.** Cyclin- D1/E/B1 co-expression (Bar size: 10 μ m); **e.** 1cen signals with *MPs* (G1/S/G2); **f.** 1cen signals with *MPs* (mult -S phase); **g:** 1cen with *MPs* (2G1/2S); **h.** Cyclins E/ B1 co-expression (Bar size: 20 μ m); **i & j.** 8cen presents *MPs* (4G1/1S, 2G1/2S, 5G1/1S); **k.** C-MYC/PE with mosaic expression; **l.** Monosomy and hyperdiploidy of Cen 1. The single signals reflect G1; the dual signals reflect G2 and triangle signals present S-phase.

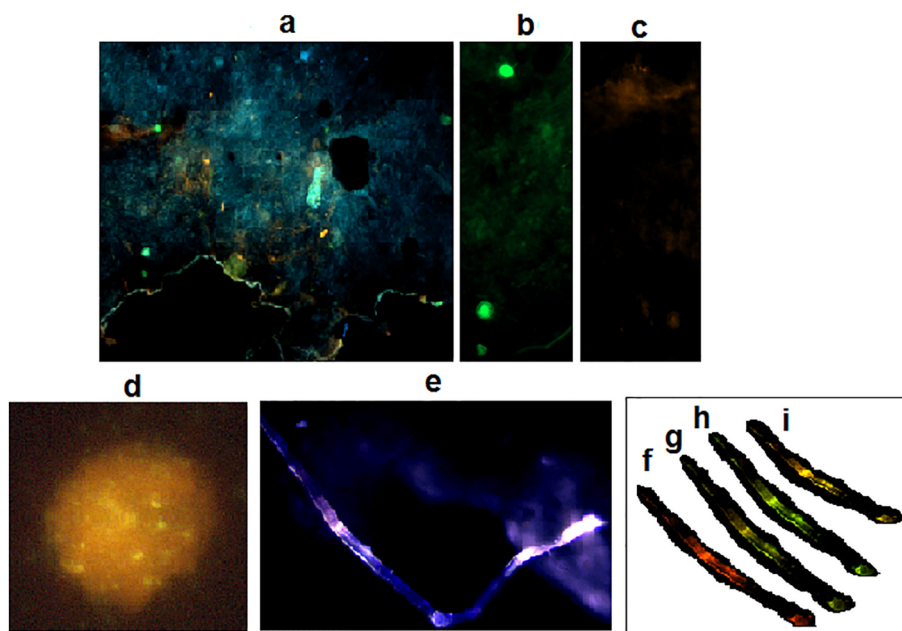


Figure 5: Protein expression and co-expression of CD44, CD24, cyclin E, B1 and Ki-67 and status of signal copy number of chromosome 3cen in breast tumor.

a. CD44/CD24 co-expression; **b.** PE+ of CD44 (FITC) (Bar size: 10 μ m); **c.** PE- of CD24 (Rpe); **d.** SCN of 3cen with *MPs* (6G1/1S) (1000x); **e.** Cyclins E/B1/Ki67 Co-expression in CTC (vasculature); **f-i:** cyclin B1/Ki67; cyclins E/ B1; cyclin E/ Ki67; & Cyclins E/B1/Ki67 co-expression. Bar size: **a:** 20 μ m, **b-c:** 10 μ m; **c-i:** 20 μ m.

The single signals reflect G1; the dual signals reflect G2 and triangle signals present S-phase.

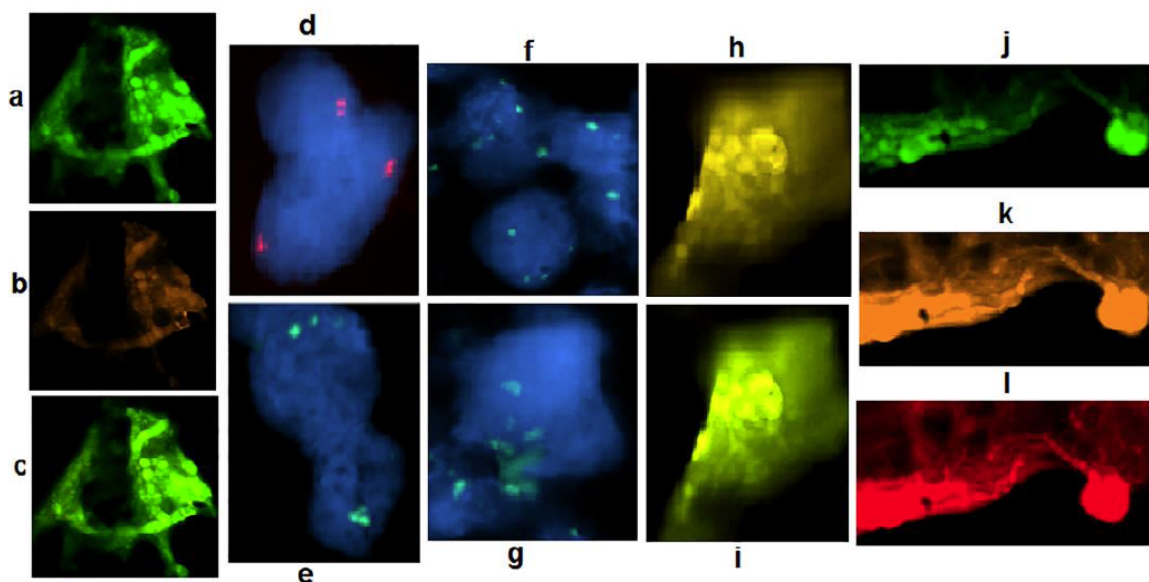


Figure 6: Mosaic phases and protein expression of CD44/CD24 in a breast cancer patient.

j-l: Total harmony for PE in cyclin B1 & CDC25A (in **k,l**); Cen: centromere; **a,b,c:** CD44+, CD24- & merged image -: +stem cell (Bar size: 10 μ m). **d-g:** 3cen-aneuploidy in G2, G1 (**d**); 1cen aneuploidy in G1 (**e, f**; magnification: 1000x); **g:** MPs as G1/S/G2. **h & i:** Cyclins E/B, & cyclin E/PCNA co-expression (Bar size: 20 μ m). **j-l:** total harmony for PE in cyclin B1 & CDC25A (**k,l**). Cen: centromere.

The single signals reflect G1; the dual signals reflect G2 and triangle signals present S-phase (Images **d** and **f**).

the personalized inhibitor-based therapeutic strategy would be paved by inhibiting the forward passing and/ or stimulating the backward movement of the cell cycle.

Lacking *MPs* was observed for chromosome 3 (in 23 patients); and equal value for chromosomes 1 and 3 (in 2 patients).

A harmonic cooperation between cyclin B1/CDK1 indicated the successful cell proliferation and the onset of M-phase. Conclusively, for a successful course of evolution, 30 combinations of different phases were traced (Figure 1).

Diverse *MPs* pattern is remarkable and reflects the personalized profile for each patient and chromosome, but evaluation of total data provides a global insight. Besides, more frequent *MPs*' involvement is, respectively, related to G1 and S for chromosomes 1 and 3 ($n=97,68$); G1,G2 ($n=47,29$); and in spite of the low frequency for chromosome 3, it is followed by G1,S,G2 ($n=49$) and G2,S ($n=12$). As the matter of fact, mosaic event for G1/S phases is also an initial required step for the successful cycle in cancer, followed by G1/G2 and G1/S/G2 complexes (data not shown). Interestingly, it seems that S/G2 and G1/S/G2 complexes have occurred in few cells for chromosome 3. It may be stated that interaction between G1 with either- S or G2 phases in both chromosomes, is

accompanied by only few cells with S/G2 complex in chromosome 3 which may lead to a successive cell cycle process. Such pattern of *MPs* may be altered during further step of tumor progression or remains stable for more cell cycle processes. This sequential cell cycle progression, even as the minor clones, highlights the natural progressive manner of cell cycle in the cell populations including those lacking *MPs* and cells with positive *MPs*. Now the question is whether the cells in first category are really normal or in the middle way of being evolved.

The *MPs* were observable for chromosome 1 in G1/S phases, and G1/S/G2; but for chromosome 3 in S/G2 and G1/S/G2. So, the course of evolution relies on the cellular strategy in direction of forward and/or backward in the increased or decreased values for the *MPs*. The ratio indices, in chromosome 1/3 was >1 in 23 patients and for <1 in 15 out of 38 patients. These findings indicate the diverse involvement of different chromosomes in the *MPs* process (data not shown).

Beside the presence of cyclin- D, E, A and B, the function of CDC25-, MYC, growth/proliferative factor, stem cell markers, and P21 play the complementary role for an early detection of protein expression by the CTCs. The PE of cyclin E, CDC25A and Ki67 and the clinical outcomes have been published [11]. Cyclin E and CDC25A were significantly expressed in higher stratum of Ki67. There was a correlation between either

cyclin E or CDC25A expression and the Ki67 index with negative impact on survival.

In fact, regulation and the functional capacity of cell cycle rely on the machinery of PE and co-expression of the involved proteins in cancer. The architecture of cell cycle depends on the individualized strategy and the formulated platform of cell cycle.

In case of irreversibility of *MPs*' pattern, a combat phase and continuous manner of cell division with higher proliferative capacity of cells may be re-initiated. So, the cells' protein reservoir may be available leading to a more severe status of malignancy. In contrast, if they behave as the supportive platform against cancer, so, there is a beneficial manner to overcome the uncontrolled/progressive/successful cell cycles. In spite of diverse pattern of *MPs* alterations, there are remarkable similarities between the patients' groups which is convincing for performance of predictive/preventive strategies in clinical managements. This Figure is rather a puzzle in which the progression of the evolution is more complicated for patients in the 1st and 2nd top groups. Dual and triple co-expression between cyclins E/B1/Ki-67 are corresponding with the presence of *MPs* in G1, S and G2 in the same BT sample (Figure 5).

By satisfactory responding to regimen including chemo- and/or radio therapy, the patient may become resistant to the treatment. Such event is due to the tumor progressive evolution. Regarding the chromosome effects, mitotic modification and aberration in cancer, it was reported that alterations in CH is "Unknown" [18]. However, regarding the *MPs*-model, it seems that an unbalanced cyclic event may have a positive impact on the clinical managements. Different combinations of phases including G1/S/G2, 4xS and 2G1/2S for chromosome 1 are indicative of variation for the phases (Figure 3e, f, g respectively). The harmonic expression and remarkable co-expression between cyclins E/ B1 is provided (Figure 3h).

The signal copy number (SCN) of cyclin D1 including 2G1/S/2G2 and combination of 4G1/1S, are confirmed by co-expression of Cyclins D1/E/B1 and Cyclins E/B1 (Figure 4a, b, d, h). The exploration of CTCs would lead to an early detection and an appropriate clinical managements. Besides, the expression of cyclin E, is remarkable within the vasculature territory (Figure 4c). The harmonic behaviors between the pattern of *MP* and PE mode in the cell cycle machinery is important to be evaluated.

So, mode of PE for C-myc, diverse pattern of *MPs* for chromosome 8 (Figure 4i, j, k), and aneuploidy status of centromere of chromosome 1 is demonstrated (Figure 4L).

However, *MPs*, as micro-chimers, reflect the sequential characteristic of cancer cells. Besides, the PE and co-expression of CD44, CD24 (Figure 5a-c) and the *MPs* with 6G1/1S of chromosome 3 are presented (Figure 5d). The positive stem cell (CD44+/CD24-) even in two cells may have impact on tumor progressive behavior. The co-expression of cyclin E, B1 and Ki-67, either as dual or triangle is remarkable (Figure 5e-i). Furthermore, the harmonic functional behaviors revealed to be remarkable for three different panels including positive stem cells (CD44+/CD24-), aneuploidy and occurrence of *MPs*, and co-expression of the related cell cycle proteins (Figure 6a-l). Expression of "multiple cyclins" has been reported in *S.Cervisidae* and mammalian cells including the presence of cyclin A and B in S phase, G2, and early mitosis; or D-type cyclins and cyclin E in G1 phase [19].

The *MPs* in the individual cell population may have benefit for BC-patients, through rescuing the cells from further progression by moving backward instead of forward. It seems that there is no barrier for the *MPs* to move around through the entire cell cycle.

A novel diagnostic assay for tumor cells and its application in response to therapy is related to the 'Cellular Archeology'. So, the process of evolved phases follows the jumping manner and may be managed through the precise estimation of *MPs* for the cell-cycle-based therapy. Application of such strategy would harmonize the manner of cell response to the therapy; the duration of therapeutic outcome; the occurrence of disease free condition; and an early detection- of tumor and/or metastatic event or secondary tumor.

Some insights on *Mosaic phases* and clinical managements include somatic/genomic- heterogeneity and evolution; multi-disciplinary behavior of malignant cells; cell response to therapy; chemo-sensitivity of proliferating cells; high sensitivity of mitotic cells; mutation/functional correlation; drug resistance and its stability.

CONCLUSION

Mosaic Phases (MPs) hypothesis in the patient with primary breast cancer is discovered. *MPs* may be

considered as polymorphism; *benefit for the tumor evolutionary process; or benefit for the cancer patients.* Puzzling around the cell cycle is a guide for decision making in clinical managements/classification including the CDKs inhibitor-based therapy. Briefly, the strategic definition of cell cycle relies on the diverse *MPs* as the backward and forward processes. Nature is aimed to progress the therapeutic channels through the cell cycle in cancer patients. This is an evolutionary forward and backward.

COMPETING INTERESTS

The author disclose any financial or non-financial interests that are directly or indirectly related to the present work submitted for possible publication.

AUTHOR CONTRIBUTION

PM contributed to conceptualization and design of the study; imaging and analysis of the FISH- and protein expression; interpretation of the data; proposing and authenticating the hypothesis; writing, review and editing the manuscript.

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DISCLOSURE

The author has no conflicts of interest.

ABBREVIATIONS (CODING)

A = G1,S/G1,G2

B = G1,S/G2,S

C = G1,S/G1,S,G2

D = G1,G2/ G1,S

E = G1,G2/ G2,S

F = G1,G2/G1,S,G2

G = G2,S/ G1,S

H = G2,S/ G1,G2

I = G2,S/G1,S,G2

J = G1, S, G2/ G1, S

K = G1, S, G2/G1, G2

L = G1, S, G2/ G2,S

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