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RESEARCH ARTICLE

Translating Single Cell Secrets to Cancer Evolution Accelerates Personalized Therapy

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ABSTRACT

Successful cancer evolution (CE) relies on the sequential molecular and functional events including 1) telomere; 2) sub-telomere; 3) epigenetic; 4-6) hit-episodes; 7) an innovative cell cycle machinery, as the multi-phase, and 8) chromosomal abnormalities. In this regard, eight available, fundamental/evolutionary and strategic key information (Evolutionary- ID) presented.

Telomere length (TL), has the fundamental role in cancer development, with serious challenges in the clinical managements. Breast cancer and brain tumor are an unresolved problem in Science and Medicine. Besides, an early and translatable diagnostic- prognostic-predictive platform, by considering the targets-ID, is required. Diverse TL in two cases affected with astrocytoma with grade IV, revealed to be 12500 and 15000 bp in tumor, and 10000 and 9000 bp at genomic level. Interestingly, TL is declined in the lymph node, i.e., occurrence of evolution.

Sub-telomeres (STs) through the cellular journey, are the neighboring destination at genomic and somatic level. The evolutionary pattern of STs has not been, routinely, decoded to the personalized clinical managements. The ST**-**sequences, are diversely predisposed to variety of environmental factors and play influential role in healthy individuals and the patients. An early detection is available by analysis of the ST- hybridized signals in the biopsy of auxiliary lymph nodes (ALN), and/or by circulating tumor cells (CTCs) into the blood stream. Diverse pattern of signal frequency and intensity in individual chromosomes at both somatic (ALN) and genomic (lymphocytes) levels were remarkable. The most common involved targets included chromosomes 5 and 9, 16 and 19; with diverse intensity at p and q chromosomal arms respectively. These findings have the predisposing, and an initial influence through the patients' course of disease.

ST- signals, by providing the STs-ID, offer periodical and predictive, indices in cancer screening and therapy.

Furthermore, the complementary, cell cycle protein expression (PE) including Ki67, cyclin D1, and cyclin E, accelerates an early clinical management through the period of disease based on the CTCs.

Epigenetics is the next molecular destination by focusing on the genomic/somatic index, as an evolutionary Epigenetics-ID with its impact on the cancer management. The target panel is Ataxia Telangiectasia mutated gene (ATM) as the molecular marker and an initiator of different cancers.

ATM has remarkable roles, including: 1) in DNA double strand break (DSB), 2) to initiate different types of neoplastic disorders, including cancer, and 3), polymorphism, D1853N as a peridisposing marker by initiating the hit process. The influential characteristics include: family history of neoplastic disorders through the pedigree, the key role of *ATM* promoter methylation, cooperation of ATM/Rb protein expression, D1853N- *marker*, telomere length (TL) and the clinico-pathological characteristics in different types of brain tumors, and the environmental factors. Interestingly, TL has an independent influence on the progressive cancer evolution. An early detection by CTCs based on the D1853N/Sub-TL/Cell cycle checkpoints based on the PE assay and molecular test facilitate an early detection and therapy, based on the personalized approach.

By highlighting the preventive insight in Medicine, a brief record on the "**Methylation in Chorionic villus samples** (**CVS**)" with aim of an early detective strategy is provided. All nine CVS samples were methylated for the MCPH1 gene. An early detection is possible either through CV sampling or by the circulating CV cells in the maternal blood.

Evolutionary Hit includes: presence of D1853N polymorphism of ATM, as the hit-initiator through an evolutionary and progressive molecular based sequential alterations led to discovery of three-hit hypothesis in a patient affected with astrocytoma. More hits include five, and eight- hit hypotheses in primary breast cancer patients. Such platforms are considered as the individualized model in cancer. The pedigrees and details at the molecular follow-up studies and functional alteration at protein level are available in the provided sections.

Novel strategy of Cell cycle phases in breast cancer is the major intersection for cancer therapy.

The novel cell cycle hypothesis (CCH) highlights the mosaic based of dual and/or multi-phases, as minor clones at single cell level in the breast cancer (BC) -patients, escorted by the normal cell population. Such mosaicism provided an archetypal, unique diagnostic and therapeutic model, by applying different mosaic patterns (*MPs*) as well as "G1/S, S/G2 and G1/S/G2, and accompanied by normal phases, as a sole including G1, S, and G2 at the single cells level.

Diagnosis is based on the mode of signal copy numbers (SCN) and the related PE. Interestingly MPs were also unmasked in patients with chronic myelogeneous leukemia and other solid tumors.

Finally, the *predisposing/predictive/prognostic/preventive* square provides an innovate CDKs inhibitor-based therapy in BC and other cancers.

Personalized base cancer therapy is the confusing procedure and requires the pedigree-based data, personalized, evolutionary based information including molecular and functional at both genomic and somatic, at single cell level. The target territories comprise cell cycle phases, proteins, Telomere length, telomerase, sub-telomere, and Epigenetics. The aim is directing the cell cycle fundamental forces back to normal, by performing:

1) Applying personalized, single cell-based approach, at molecular, functional level, pedigree analysis, and balancing the micro-/macro-environmental factors, including nutrition.

2) Satisfactory high single cell enumeration based on the FISH and protein expression assays;

3) Decoding the required dosage and combined therapeutic regimens accordingly,

4) Unmasking the cell cycle combined (mosaic) phases including different Cyclins; and

5) Bilateral cooperation between Pharmacology, Medicine, and Cancer Genetics/cell biology.

Let's combine the *evolutionary based strategy by translating the personalized data at molecular/ Functional/ Informative, and pedigree-based level to the personalized therapy*.

Keywords: Breast cancer; brain tumor; Auxiliary lymph node; Telomere; Sub-telomere; Epigenetics; Evolution; hit; Cell Cycle; Hypothesis; Personalized; cancer therapy.

General Introduction

Health is a puzzling standard. Initiation of neoplasia and progression passing through the developmental gates, including all the essential harmonic channels, up to cancer creation:

1) Through the somatic, and sequential events; the initial sign of alteration is, hidden through the human's cellular territories; at first week and/or during the embryonic and fetal development. They may, hibernate approximately 10 years until an initial clinical manifestation. Due to rapid division, growth and the cooperative behavior, the initial clinical management is, extremely, late.
2) The evolutionary and progressive events have negative impact on cancer therapy and the patients' quality of life, the family and society.
3) Considering the micro- and macro-environmental factors including nutrition.
4) It is essential to consider: a) the key role of molecular events in cancer initiation and progression, b) the nature of molecular and functional alteration, and unmasking the course of evolution through the target patient's life.

The biological landscape in cancer depends on the somatic/genomic diversity; evolution; multi-territorial commandments, including Telomere; sub-telomere; epi-somatic /epi-genomic: methylation; hit-events; and cell cycle machinery. Besides, the selected mentioned events are cooperative as well. At a glance, a brief history of cancer scenario includes, important role of mutation in cancer, by highlighting Rb gene, unmasking the hit hypotheses; bolding the predisposing role of ATM in variety of tumors; unvieling the hit- hypotheses in the neoplastic disease including cancer.

1-Telomere

Telomeres, are the edges of the chromosomes' ends, and defend them against deprivation;
play the fundamental role in joining the chromosomes' ends to the nuclear envelop. Telomere length (TL) is categorized by telomerase activity; telomeric association; cell division; and fusion [1,2]; chromosomal stability and replication[1,3]. Besides, telomeres are characterized with tandem hexametric repeat (TTAGGG)n. The most of apparently normal somatic cells lose approximately 50-100 bp of the terminal telomeric repeat DNA at each division of cell cycle [4-8].

Telomeres' shortening control the normal cells' proliferative capacity and aging [5-7]. Telomere shortening is performing as: 1) a block to impose limitation on the cellular division; 2) stabilize the cellular aging and the lifespans period; and, organize the cells derive to be senescent [9-11]. The applied techniques included high pure DNA extraction, Southern Blot, analysis of TRF Length, by considering the provided classifications [8,12,13]. Exploring TRF Length is performed in 50 brain tumors' (BT) samples, based on the WHO grading [14]. It was reported that: 1) the TRF length of brain tissues was not reduced by age and varied between 9 and 13 kbp and between 9.4-13.2 kbp [14-16]. Furthermore, our results revealed a high significant difference between TL in meningioma and astrocytoma with normal range ($p<0.001$). Conclusively, by considering the patients' age, the malignant tumors with short telomere length and dysfunction are catagorized as a negative prognostic marker in solid tumors, including breast [15, 17-19].

Highpoints:

-The initial discovery of telomeres' structure reported 85 years ago, [20].
-The structure at the end of individual chromosomes are reported within 1941s [21].
- Telomere made of six nucleotides with heterogenic pattern in different organisms, within 5–10 kb in humans and shortening of telomere length (TL) was reported in human fibroblasts [22,23].
-Structure and variation of the chromosomal ends has been announced [24].
- Reduction of telomeres was proposed in colorectal cancer[25].
- The survival in B-cell chronic lymphocytic leukemia was related to the Telomere length and telomerase activity [26].
- Shortening of TL in granulocytes, as : a diagnostic marker for myeloproliferative disease is Reported [27].
-Expression of hTERT mRNA is related to telomerase activity in human breast cancer [28].

Heterogeneity in breast cancer and brain tumors

Materials and methods

1)DNA extraction from the tissues by high pure PCR template preparation kit; 2) Standard protocol of Telo TTAGGG Telomere Length Assay kit, as a non-radioactive chemiluminescent assay, was performed; 3) protein expression assay assayed by immunofluorescence; 4) performing two functional based methods include Q-FISH and protein expression [19-28].

Q-Fish

High enumerated cells including 28889 in peripheral blood and 45803 cells in tumor of patients affected with primary BC are performed by Q-FISH. The same manner was applied for the patients affected with primary BT.

Differences of SI scoring between tumor samples and their corresponding blood in BT and in BC patients are presented.

Cell cycle protein analysis

The low level of PE for cyclin E, c-fos and Ki-67 was observed in the BT samples. The significant correlation between expression of cyclin D2 and cyclin E in BT cells; and significant correlation between cyclin E and c-fos were remarkable.

Heterogeneity in Telomere length

Heterogeneity of Telomere length, reflects the cellular evolution, at genomic and somatic level [19, 43-45].

Evolutionary hypothesis of TL, was initially unveiled in primary BC and brain tumors (BT) to provide an unique marker for cancer clinical management including prognosis, prediction and planning for an early detection by the circulating tumor cell (CTCs). The results of genomic/tumor ratio included G/T of TL in patients affected with primary BC and primary BT revealed significant differences. By application of the Q-FISH, telomeric signals echoed the significant decreased in the tissue to blood, either in BC or BTs, reflective of an evolutionary event in TL during the development and progression of cancer.

The following findings unmasked the heterogenic and personalized pattern of TL by considering G/T diversity:

Heterogenic pattern of TL is traced:

1- In the tumor and blood samples in BT patients.
2-In BT with low histopathological grades, and very short TL was detectable. In a grade I meningioma tumor, the TL-length revealed to be 0.940 and 8.600 kb in tumor and blood sample, respectively
3-In a grade II astrocytoma tumor, the long TL(16.000 kb), versus her blood TL (8.800 kb) was remarkably heterogenic.
4- The TL in tumor was shorter than the lymph node sample.
5- TL of breast tumors was significantly shorter than brain tumors
Expression of hTR and hTERT in human breast cancer was correlated with clinico-pathological parameters[29]. Furthermore, telomerase activity and expression of its RNA component (hTR) was correlated with clinico-pathological characteristics; it was also highlighted that the association between hTR expression and younger age facilitate for implicating a telomerase gene-based therapy in younger women (<40 yrs) [30]. Furthermore, brain is highlighted as the favorite metastatic destinations from the breast in which the involvement of ATM, as a predisposed marker is found to be a risk factor [31]. In this case, the consideration of preventive strategy is required.

Shortening of telomeres is reported during the aging period of human fibroblasts, [32]. Furthermore, telomerase expression is a reliable marker in the clinical management of patients with lymph node metastasis [33]. This strategy is applicable by exploring the presence of the migrated cells to the blood stream of BC-patients, as a non-invasive, rapid, and repeatable test in only 2 ml of the patient's peripheral blood or a touch sampling of buccal smear.

The remarkable diverse TL-ratio of G/T in both group of patients, highlights the evolutionary hypothesis of TL in cancer progressive developmental period. However, TL in brain was more heterogenic than in breast tissue.

The up-regulated Ki67, cyclin E expressions were associated with clinical follow-up data in breast carcinomas which were confirmed by flow cytometry and manual immunofluorescence (**Figure 1**).

In spite of low expression of CDC25A (b), up-regulated protein expression of Ki67 (c), and Cyclin E have dominantly proceeded the progressive proliferation with successful cell cycle machinery-manner of the patient's breast.

At a glance, the hypothetic and evolutionary pattern in TL is reflective of the genomic–tumor heterogeneity and instability in breast cancer and brain tumors.

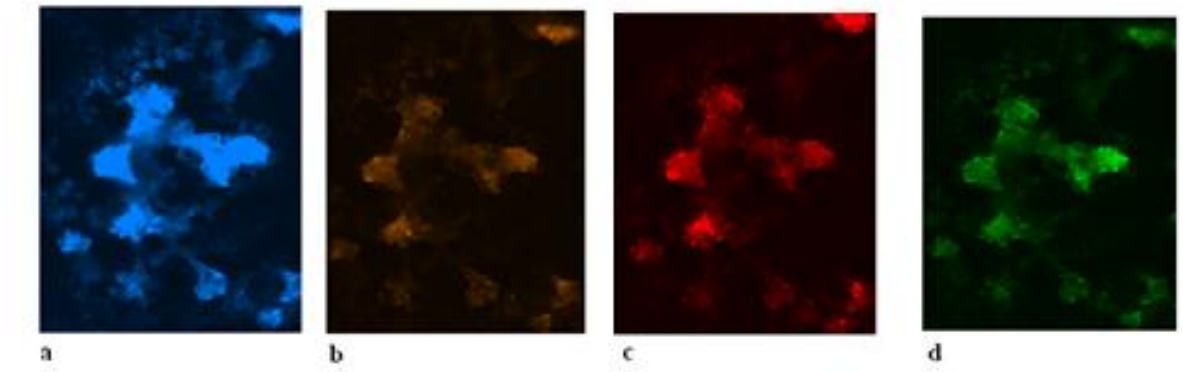


Figure 1. Protein expression of Ki67, cyclin E and CDC25A expression in breast cancer cells
Breast tumor cells conjugated with, a) dapi presents breast cells; b) same cells with R-pe/CDC25A, lacking expression; c) same cells conjugated with pe-cy5/Ki67 with high expression; and d) same cells with FITC/cyclin E. Bar:20 µm

Conclusions:
Valuation of the cyclic proteins, and the TL assay, open the cooperative and functional insights in cancer development, progression, and early clinical management.
In spite of the available worldwide data in variety of cancers, including BC and BT, an early- detection and therapy is unavailable, especially based on the cell cycle.
Furthermore, the function of TL-diversity at genomic–tumor level, and telomerase, are, considered as the key prognostic elements.

2.Sub-telomeric Signals
Introduction
Breast cancer (BC) is the most common cause of cancer mortality. It is required to challenge the cellular events, based on an early detection, and translating to the clinical management. In the early 1970s, it was unmasked that chromosomes are, unable to replicate their terminal regions [11]. It was also, recognized that the DNA sequences are loosed during each cell replication, sometimes at maximum level up to the termination of the cell division [35, 36].
The terminal section of chromosomes formed by telomere repeats followed by subtelomeric (ST) sequences, consisting of repeated sequences [35]. However, by emphasizing on the repeated genes, TL and ST are a combined section which formulate and fate the cellular evolutionary model [37-39]. It has been also reported that diverse TL in cancer cells play a directive role in cancer progression. Besides, the genomic and somatic evolution, diversity, and heterogeneity in BC and BT, by aiming the individual *p* and *q* chromosome arms, have been previously published [24]. The frequency and nature of the related lymph nodes (ALN) are applied as a supportive and diagnostic tool in BC management [38].
The quantitative-FISH analysis was performed in the individual chromosomes [39,40]. The challenges include, remarkable diversity, discrete evolution, tumor heterogeneity, and aging, leading to the major and minor STs differences in the cells. By considering the severe heterogeneity in each cancer type, measuring the global TL is insufficient. Therefore, ST analysis by FISH will deliver translatable data for predisposition, prognosis, prediction and personalized clinical managements.
The housekeeping genes were also evaluated for analysis of gene expression [41], and redirecting T-cell specificity for human applications have been also reported [42]. Association of telomere/ telomerase /STs, unmasked the diverse copy number of subtelomeric sequences of buccal and ALN cells as a personalized characteristic for the breast neoplastic progression [43-45]. Furthermore, the ALN's subtelomeric characteristics could be assayed by circulating tumor cells (CTCs), as the personalized follow-up study and an early detection of the development of any malignant lesion in the target individual (**Figure 2.**).
The results are summarized and provided figures demonstrate the following themes [43-45]:

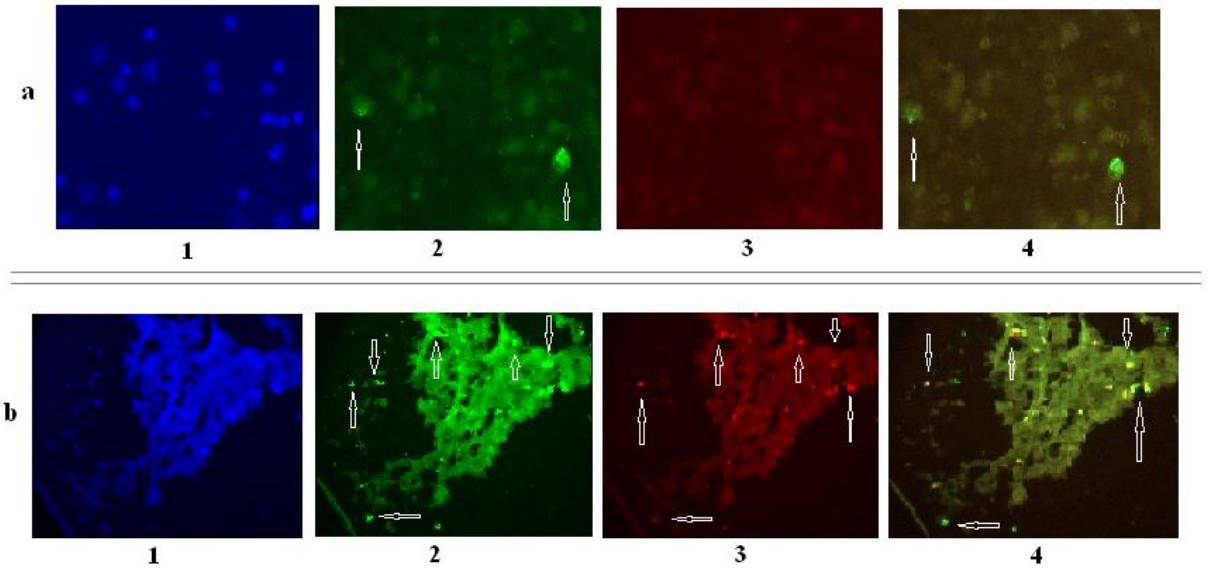


Figure 2. Sub-Telomeres signal copy numbers and signal intensity in ALN and genomic cells for chromosome 1

a. Cells at genomic level, unaffected cells from the same patient, two arrows refer to the circulating tumor cells to the blood stream.

b. Neoplastic cells in auxiliary lymph node (ALN).

A typical labeling of ALN and, with specific probes for the p and q arms of chromosome 1. Analysis with all probes showed that cells with positive signals correspond to 11.21% (4506/40.197) for ALN and 28.43% (2415/8494) for unaffected genomic cells.

Adapted from: [45]

Two cell populations, including normal and abnormal cells, were analyzed in order to assess their heterogeneity. The SCN and SI of FISH were used to characterize the STs presence in each or all chromosomes and to distinguish the *p* and *q* arms. Highly significant differences in the labeling of each chromosome ($p < 0.001$) by comparing the status of ratio of p/q for SCNs were found in the *p* and *q* arms of ALN and lymphocytes.

Specifically, the mean value for SI of *p* arms of all chromosomes in genomic cells were also significantly higher than in ALN cells for weak and medium SI values, but not in cells with strong SI value. *Such finding is a remarkable and valuable sign for leading the clinician to aim an early detection by circulating lymph nodes cells (**CLnc**) by avoiding lymphoectomy dissection and saving the immune support of ALN.*

In cells with either one or two SCNs, the mean value in genomic cells was also higher than in ALN cells. In contrast, the mean values for three SCNs in ALN cells were higher than in genomic cells for half of the chromosomal set. As an example, the image of chromosome 20 is provided (**Figure 3.**). For few SCNs, the mean value in ALN cells was higher than in genomic cells for each chromosome.

In order to provide the expression and co-expression pattern, the categorization.

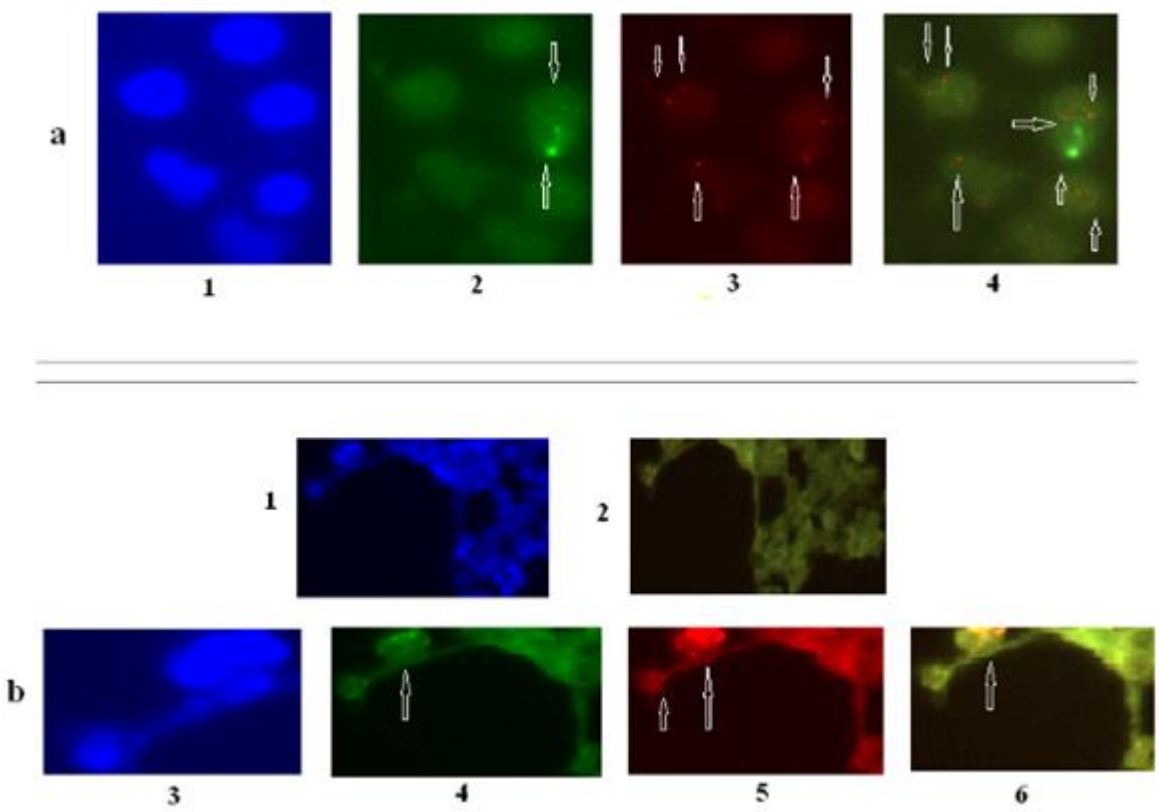


Figure 3. Subtelomeric signal copy number and signal intensity of the *p* and *q* arms of chromosome 20 in the genomic and auxiliary lymph node cells of a patient with breast cancer.

a) in blood (genomic); b) in auxiliary lymph node (ALN)

Adopted from Ref [45].

a: G cells with dapi filter (blue); Same cells are conjugated with FITC (green=p -arm) showing one remarkable signal accompanies by two low SCN and SI; a3, Same cells with pe-cy5 (red= q-arm) are characterized with higher SCN than ALN and low SI; Merged image of p- and q- arm reflecting diverse co-signaling of p and q arms.

Middle and bellow rows, b: ALN cells with dapi filter; Merged cells conjugated with FITC and pe-cy5 without SCN and SI for both arms is visible.

Bellow row: ALN cells with dapi (blue); cellsconjugated with FITC for p arm with Lower SCN and SI than q-arm; same cells with Pe-Cy5 for q-arm presenting higher SCN and SI than in p-arm of cells with brilliant intensity; Merged image of p and q-arm, presents higher intensity in q - than in p - arm; and dominant strong intensity of q-arm and SCN.

ALN: Auxiliary lymph node; G.: genomic; S: somatic; SCN: Signal copy number, SI: Signal intensity

Cell magnification: x1000 (top and bellow rows); x400 (middle row). Cell magnification: x1000 (top and bellow rows); x400 (middle row)

Bar size: middle row and bellow row: 20 µm

Human telomeres anchorage six tandem repeat (TTAGGG), accompanied by the complex subtelomeric field [46, 47]. The genomic/somatic evolutionary hypothesis has provided a new insight in cancer evolution [24].

Occurrence of evolution in telomeres- and sub-telomeres' at genomic and somatic levels, highlights the cooperatve machinary at the chromosomes, and occurrence of the heterogenic pattern in human neoplastic disorders [44]. Furthermore, the environmental factors affect STs by initiating and progression of the neoplastic disease through the evolutionary course [46].

However, the total telomere length and status of telomerase activity at genomic and somatic level play role in early clinical management [42].

At a glance, the specific and personalized pattern of Tels and Sub-Tels in the target individuals

affected with neoplasm, or in apparently normal individual with family history of any neoplastic disorder (s), lead to categorize heterogeneity at the genomic and neoplastic levels. Detection of such profile at genomic level could be considered as an alarm for early detection, prediction, and planning an appropriate management at the right time. The individualized heterogenic pattern at the genomic and/or ALN levels provide the early and predictive information for considering the early clinical planning through the patient's life.

Conclusion

By highlighting the notable link between the telomeres, subtelomeres (ST), and the behavioral instability, the non-invasive sampling and assaying the peripheral blood, and/or buccal cells are an initial step for further assay at the neoplastic level. These results reflect the individualized evidence for the complementary test, and also for the predisposed family members within the pedigree.

Analysis of telomeric and subtelomeric profiles in the peripheral blood and/or buccal cells, as the non-invasive and available samples are the reliable controlling for an early detection either for the proband or for the targeted relatives. Such strategy guarantee the Predictive, Prognostic and Preventive plan for an early detection within the whole pedigree.

3. Epigenetics

1)Epigenetic with its three-dimetional and diverse patterns within individuals reveales to have diverse functional format of DNA methylation [53]; **2)** Epigenetics, with the tri-dimensional characteristics play the key duties within the ancestral line; **3)** Therefore, planning for the prenatal screening of fetal DNA is possible by screening the maternal plasma [54]. **4**) Furthermore, tracing the etiology of cancer [55]; the p53 reversing phenomena in multiple melanoma is notable [56];

Epigenetics is rather a complicated destination in cancer, and the mechanisms of methylation harbors the multi- intricate characteristics. Where does the tragedy initiate? The events rather recruit from the embryonic passage, although the roots are the parents, but with very complicated function (s). It was theoretically proposed that the parents harbor the imprinting genes with limited methylation and expression pattern in each inherited alleles [55]. These genes are, selectively, methylated in maternal or paternal allells, which have decisive roles in the growth of fetus and placenta, plus have the influential impact on the fetal neurologic and behavioral development. The interaction of *RASSF1,* interact with the XPA protein by up-expression of cyclin D1 and arresting the cell cycle. The *RASSF1,* as a differentially gene has significant roles in DNA repair system by interacting with XPA protein. It is involved in suppression of cyclin D1 and arresting the cell cycle, by representing its role as a tumor suppressor gene [56-58]. In addition, this gene, by dual action is: 1) hypermethylated in placental tissue; and 2) becomes hypomethylated at the post-birth/ neonatal period [59].

4. Hit-Hypotheses

Ataxia Telangiectasia mutated gene (ATM)

The predisposed/predictive/preventive/prognostic insight (4XP) is the characteristics of D1853N marker. Thereby, a patent is registered (Mehdipour, 2014; PCT/IB2014/065072) in which the role and applicability of D1853N as a 3xP cancer marker in the majority of solid tumors and leukemia's, are highlighted. Besides, the further sequential - molecular and the expression of Ataxia telangiectasia mutated gene (ATM) or other relevant tumor suppressor gene/proteins, may play the fundamental role in tumorigenic progressions.

4a-Two-hit hypothesis

The molecular hit-events may occur, but so far, very limited hits are discovered. Knudson, initially, reported the two-hit hypothesis in retinoblastoma gene (Rb)[60]. In spite of an unavailable and limited molecular and functional technology, Knodson has unmasked an important finding whose discovery has been supported by the informative pedigree-based analysis [60].

4b- Three-hit hypothesis

Furthermore, more-hit events in another tumor suppressor gene, i.e., ataxia telangiectasia mutated gene (ATM) has been unveiled as the novel three-hit hypothesis in a 28 years old female affected with Astrocytoma [61]. Tracing such events required to ladder the patient's pedigree. Moreover, the environmental factor (s) play the influential role in carcinogenesis as well.

The D1853N polymorphism in ATM gene, played an initial role in cancer evolution. Surprisingly, the proband's parents had no sign of this variation. Besides there was no publication available. Interestingly, D1853N polymorphism has been arisen at initial week of embryogenesis (**Figure 4**). This polymorphism, has been initially, unmasked as an ATM- predisposing factor in cancer. Briefly, the D1853N polymorphic is a predisposing factor for cancer initiating[62].

4c. Five- hit hypothesis

More hypothetic event in ATM gene has been traced in a patient affected with invasive ductal carcinoma of the left breast cancer. The cascade of

five- hit events in ATM gene included the complementary molecular alterations to the three-hit evolutionary hypothesis (**Figure 5**) [63].

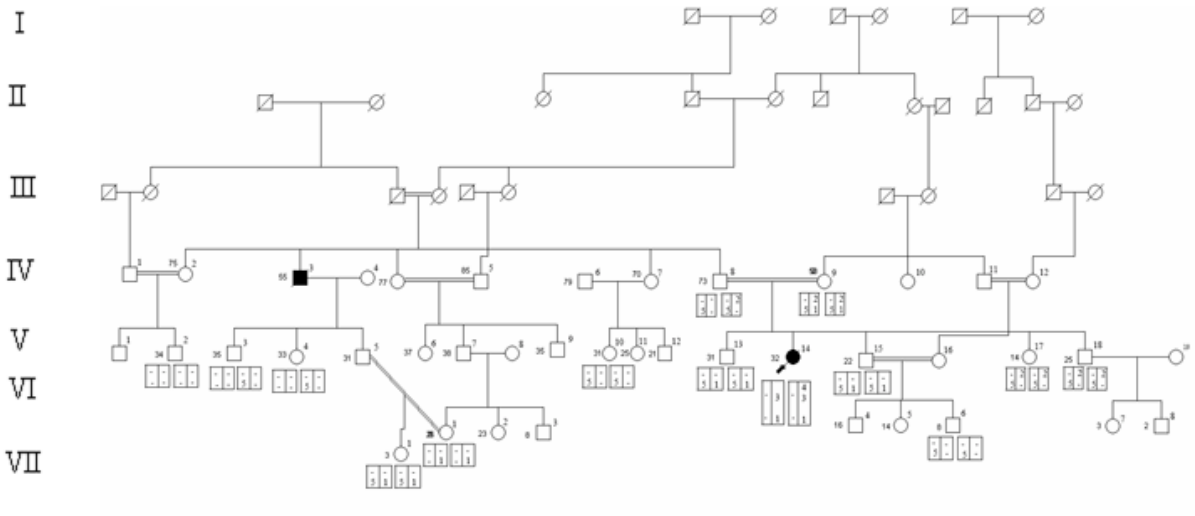


Figure 4:
Left main box illustrates information on peripheral blood sample.
Right main box illustrates information on buccal smear sample for healthy relatives and tumor sample for proband.
Column right/top of each main box presents alteraions of the 3' splicing site of intron 18. Column right/bottom of each main box presents alteraions of exon19.
Column left/top of each main box presents alteraions of the 3' splicing site of intron 38.
Column left/bottom of each main box presents alteraions of exon 39.
Left –side numbers of each individual presents age of healthy relatives at the time of sampling and age of deceased for two affected persons in the pedigree.
Right-side numbers of each individual presents systematic reference number of individuals through each generation

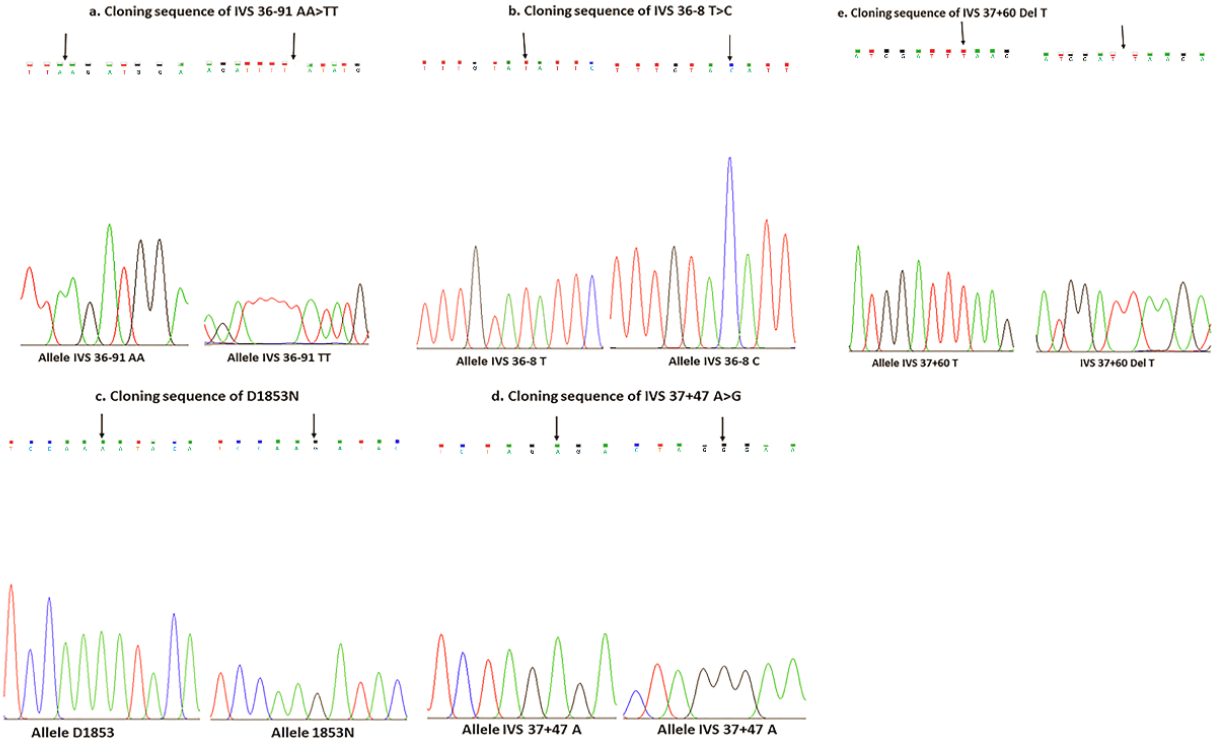


Figure 5. Sequencing of clones containing target region of ATM at genomics and tumor levels in a patient affected with primary BC

a. Cloning sequence: IVS 36-91 AA>TT (left, Allele AA; right, Allele TT); b. Cloning sequence: IVS 36-46 C>T (left, Allele C; right, Allele T); c. Cloning sequence: IVS 36-8 T>C (left, Allele T; right, Allele C); c. Cloning sequence: D1853N (left, D1853; right, N1853); d. Cloning sequence: IVS 37+47 A>G (left, Allele A; right, Allele G); e. Cloning sequence: IVS 37+60 Del T (left, Allele T; right, Allele Del T)

Protein expression of Cyclin D1, Cyclin E, and ATM in the tumor cells of a breast cancer patient is provided (**Figure 6**).

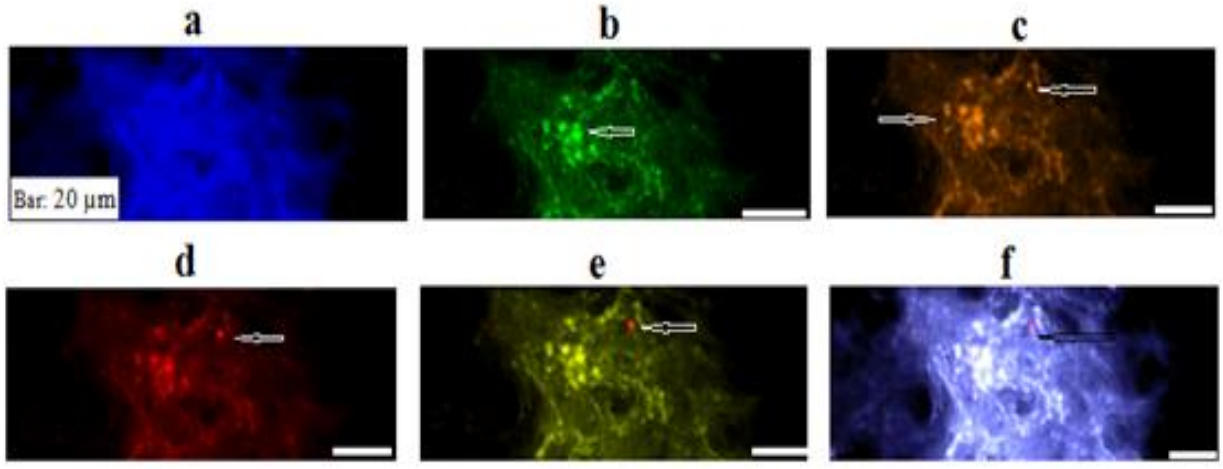
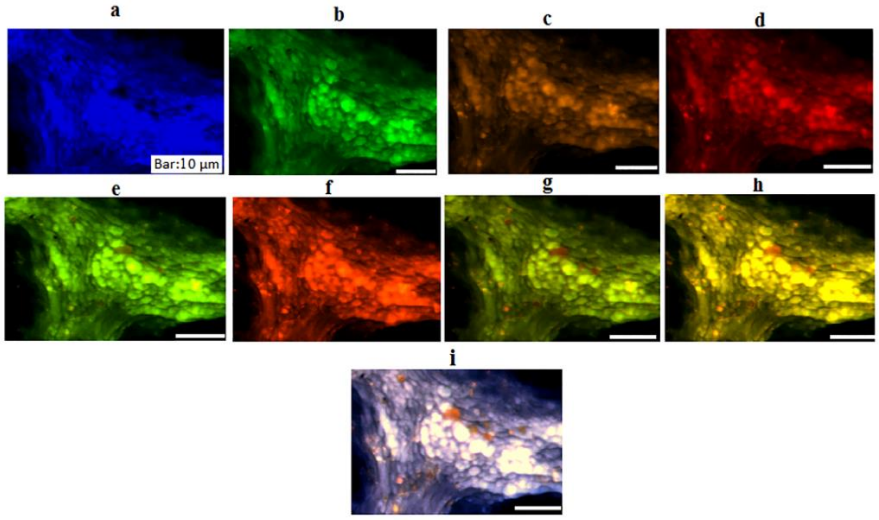


Figure 6. PE of Cyclin D1, Cyclin E, and ATM in the tumor cells of a breast cancer patient. a) Breast tumor cells with dapi; b) The same tumor cells conjugated with FITC presenting expression of cyclin E1; c) The same tumor cells with Rpe reflecting expression of cyclin D1; d) Same cells showing expression of ATM conjugated with Pe-cy5; e) Co-expression of cyclin E/cyclin D1/ATM; and f) Merged image of dapi/ cyclin E/cyclin D1/ATM. Arrows refer to the remarkable PE of ATM and its co-expression with cyclin D1. Bar size: 20 µm (adopted from Ref. **63**).

4d- Eight- hit hypothesis

Eight-hit hypothesis has been proposed based on the broader molecular and functional alterations at genomic and somatic levels in a breast cancer patient. The complementary alterations in addition to the Five -hit events included the complicated and progressive molecular changes including three more hits [64]. Protein expression of Cyclin E, ATM and CDC25A in the tumor of a patient with breast cancer is provided (**Figure7**).



Figures 7 "PE of Cyclin E, ATM and CDC25A in tumor of a patient with breast cancer".
Figure 7 is indicative of the harmonic PE and co=expression [64].
a) Breast tumor cells with dapi; b) The same cells conjugated with FITC, reflecting expression of Cyclin E; c) The same cells conjugated with Rpe, reflecting the expression of ATM; d), The same cells presenting the expression of CDC25A and conjugated with Pe-cy5; e) The co-expression of Cyclin E/ATM; f) The co-expression of cyclin ATM/ CDC25A; g) The co-expression of Cyclin E/CDC25A; h) The co-expression of Cyclin E/ATM/CDC25A; i) The co-expression of dapi/Cyclin E/ATM/CDC25A. Diverse expression and co-expression is observable.

All three evolutionary hits events have been, functionally, approved by the protein expression assay (**Figures 7**).
Three-, Five-, and eight-hit, had the evolutionary – based elements, the initial ATM involvement and sequential molecular alterations in common.
Moreover, the micro- and/or macro-environmental factor (s) played the influential role through- the pedigree, embryonic, fetal, childhood, and the adulthood of the individual's life style of the ancestral lines.

4d. Conclusion

Evolution paves the ways towards translating the cascade events to the required strategy, by focusing on the Personalized/Predisposing/ Predictive/Prognostic/Preventive (5xP) approach, and individualized therapy in cancer.
Discovery of *Hit*-events have, virtually, highlighted the D1853N polymorphism, as a predisposing marker in initiating the neoplastic disorders, and announcer for the early detection of cancer.
A complementary insight in the hit-portents will be reliable by performing protein expression assay. This strategic panel provides the most trustable clinical management based on the personalized manner. *The two-, three-, five, and eight- hit hypotheses* are unmasked through the evolutionary-based multi-channels for the health benefit of the individuals- with: 1) cancer family history in their pedigrees; 2) the target individuals with non-standard micro- and macro-environmental factors, including nutrition; and 3) application of the personalized clinical managements, including early-detection and therapy. The provided approaches reflect the translatable and trustable policy between science and medicine for the patients/ benefits.

Section 5

5.1. An innovative insight of the cell cycle:

Mosaic multi-phases

The historical and scientific backgrounds have reflected the manner of cell cycle machinery, as the non-stop circling process and according to the clockwise direction. Cell cycle indorses initiation; and progression through the involved barriers.

Cancer cell cycle is characterized with speedy growth, rapid proliferation and an express cycling process. Most importantly, the evolutionary based mechanism through the barriers are planned. Therefore, balancing the single cell strategy is required.

This section provides the published data on the mosaic mode of cell cycle machinery over the G0, G1, S, and G2 phases. The cell cycle includes the controling and the transition of the related phases over the related barriers [65]. The G1/S transition includes proliferation, growth, or apoptosis; cyclin dependent kinases, cyclins and retinoblastoma (Rb1) gene [66]. The rs614367CCND1 polymorphism has functional role to influence on the regulation and expression of CCND1 gene in breast cancer (BC) [67].

5.2. The basic events through the cell cycle at a glance: [64-67]

1-At early G1: complex of cyclin D/CDK-4 or 6 is responsible for the initial phosphorylation of pRb in G1.

2- Cyclin E by activating CDK2, leads to phosphorylate pRb in late G1.

3- the cyclin A/CDK2 complex leads to the further functions in the S-phase.

4- The Cyclin B1/CDK1 complex enables transition of the G2-M.

The results reflect: 1) the functional mechanisms and targets involved in cell cycle, 2)to design the novel panel including early- Diagnostic/Predictive/ Prognostic/Preventive/early-detective, therapeutic, and personalized (6+1), periodic chart. The "Complex/*Mosaic Phases* (*CMP*)" is the translatable and personalized approach, targeting the novel combined cell cycle based therapy. However, cell cycle territory contour the scenario of cellular life including initiation, progression and therapeutic tactics of the neoplastic disorders, accompanied by the telomere, sub-telomere and epigenetics triangle.

Unmasking the heterogeneity and course of evolution in the interphase by classical and molecular cytogenetic methods, lead to the personalized, harmonized and categorical based cancer therapy in the neoplastic disorders including all type of cancer.

5.3. Materials, Methods and patients in brief

Sixty female patients affected with the primary breast cancer (BC), comprises 45 cases with invasive ductal carcinoma, three with atypical medullary carcinoma (n=3); Invasive lobular carcinoma(3) and others (n=10). The journey designated interphase. Target chromosomes included numbers 1, 3 and 8 provided as the example. The results of other chromosomal sets are available.

Cell cycle' phases were evaluated, with application of three centromere probes. Cell count for each sample included 500-1000. Discovery of the *Mosaic Phases* (*MPs*) was achieved by application of the Fluorescence In Situ Hybridization (FISH), and Flow-cytometry, at single cell level. The results were confirmed by protein expression (PE) assay with immunofluorescence. The manual based and high cell enumeration was applied at single cell level [62]. Furthermore, the biochemical motion in the nucleus occurs during interphase[63]. Each chromosome is categorized based on the presence of the enumerated chromatids in G1, S- and in G2 phases [63]. The *MPs* were unmasked by FISH analysis, and confirmed by IF; and FC [64]. The patients' clinical information, the applied evaluated and analytic systems are provided in the published data [65].

5.3.1. key protein expression of the cell cycle markers

The proteins involved in the cell cycle control of ALN cells reflect the degree of proliferation. Protein expression of Ki67, cyclin D1, and cyclin E were assayed (**Figure 8.a1-4**). Diversity in minor clones were detected. Despite the low expression of single cells, the co-expression is found to be remarkable (**Figure 8. b1-4**).

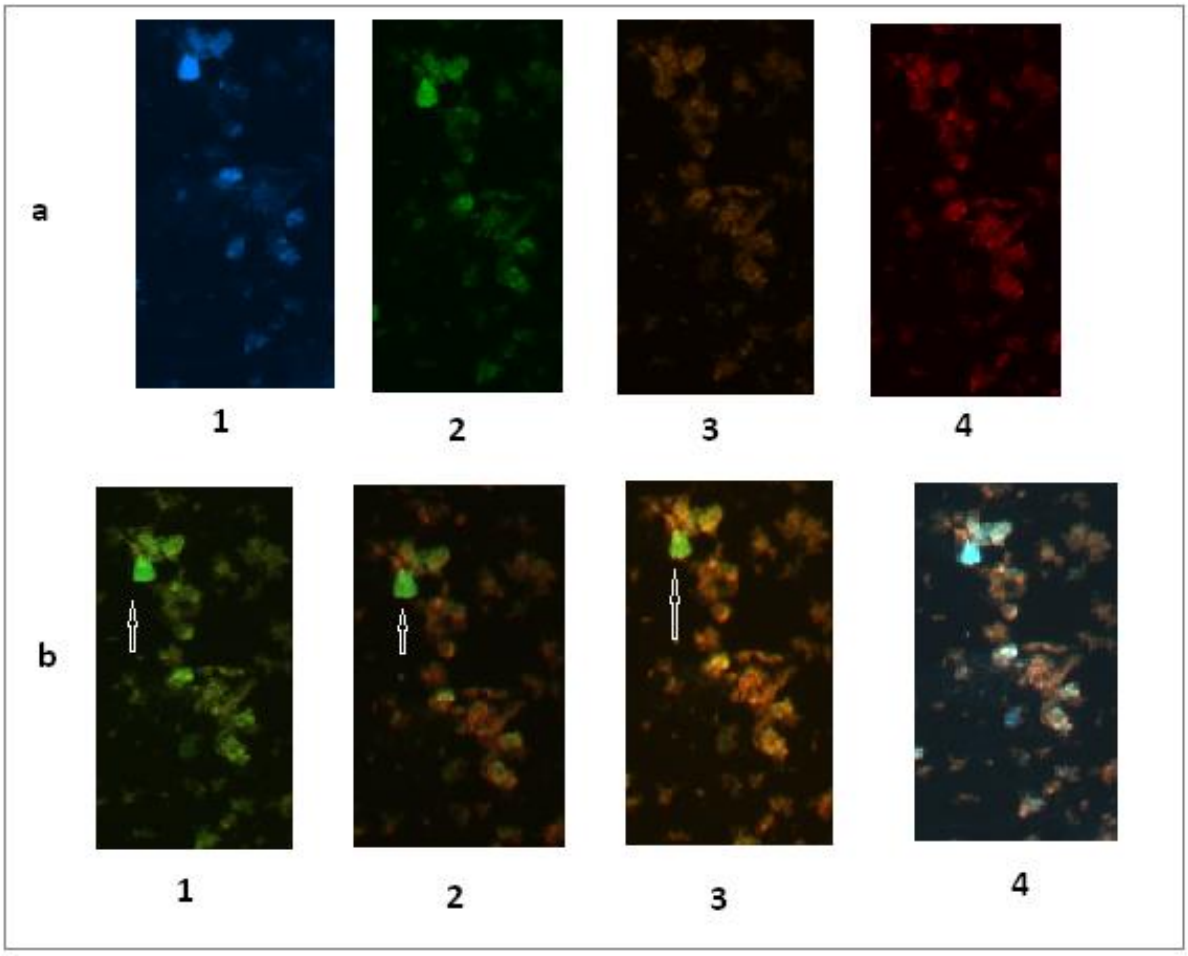


Figure 8. Status of PE Ki67, cyclin D1, and cyclin E expression profiles in auxiliary lymph node of a patient with primary breast cancer.

Row a: reflects: a1) ALN- cells conjugated with dapi; a2) same cells conjugated with FITC reflective of Ki-67; a3) same cells conjugated with cyclin D1; and a4) same cells with cyclin E.
Row B: b1) co-expression of Ki-67/Cyclin D1/cyclin E; b2) co-expression of Ki-67/cyclin D1; b3) co-expression of Ki-67/ Cyclin E; and b4: co-expression of Dapi/Ki-67/Cyclin D1/Cyclin E
In addition, detection of the PE-status would be as early as possible in the circulating ALN and cells

5.4. Strategic cellular and molecular characteristics shape cancer therapy

The *CMP* was categorized for the upcoming "multi-phases cyclic pattern/ multi-programmed-potential, and the multi-targeted therapeutics candidates" [65]. The Ratios, are calculated according to the *MPs-frequency* of the target chromosomes, by considering the A-L defined variables.
Results are provided based on the distribution of diverse signal-based characteristics of *MPs* through the cell cycle, compared with different factors as following (**Table 5.1a-c**):

Tables 5.1a-c. Cumulative distribution of survival through tertiles for variables of mosaic phases of chromosomes 1 and 3.

Only Chem- OS			
MPs	I	II	P-value
LossCh1/Loss Ch3	145±0	51±27.7	0.0246
Loss/norm Ch3	17	132.7±10	0.05
b	**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
c	**Only Chem-OS**	**Horm +radio- OS**	
Sig gain ch 1/3	90.5±38	29±1	0.048

MP: mosaic phases; Ch: chromosomes
OS: overall survival (per months)
Treated with: 2a: Only chemotherapy; **2b:** Chemotherapy; chemo- and hormone therapy; **2c:** Chemo-, radio- and hormone- therapy. [65]

5.5.Evolutionary model of the CMP

Thirty categorized data of different phases are available through the evolutionary course. The most frequent involved *MPs*-complex included G1/S, G1/G2, G2/S, and the triple form are provided (Figure **9**). The most combined form between 2G1/1S is found for chromosome 1. But, the most distribution for lacking any combined form was traced for chromosome 3 (**Figure 9**)[65].

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

CDP	Ch1	Ch3	R
3G1/1S	1	0	1/0
3G1/2S	5	0	5/0
3G1/1G2	1	0	1/0
3G1/2G2	2	0	2/0
3G1/3S	0	1	0/1
4G1/1S	6	5	1.2
4G1/4S	1	0	1/0
4G1/1G2	1	0	1/0
4G1/2G2	1	0	1/0
4G2/1S	2	0	2/0
5G1/1S	0	0	0
5G1/1G2	1	0	1/0

CDP	Ch1	Ch3	R
1G1/1S/2G2	1	1	1
2G1/1S/1G2	1	0	1/0
1G1/2S/1G2	1	0	1/0
3G1/1S/1G2	3	0	3/0
3G1/1S/2G2	1	0	1/0
5G1/1S/2G2	0	0	0

Figure 9. Frequency and ratio indices for mosaic phases for 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.
Left side numbers of each combinations presented for each phase (Frequency of signal copy numbers) [65].

Dual functional results by FISH and PE in breast tumors (BTs) are provided (**Figure 10**) [65].

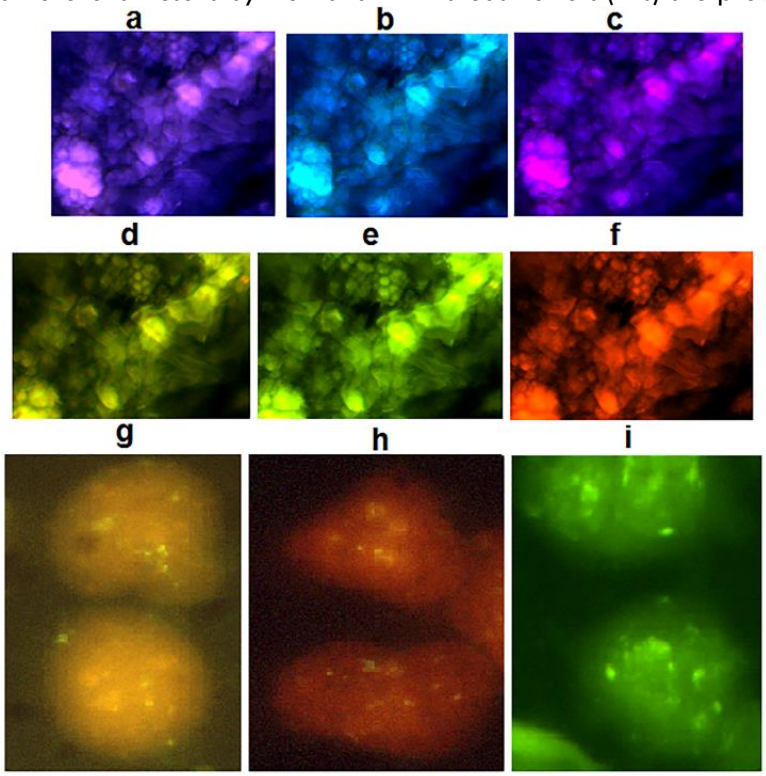


Figure 10. Status of signal copy number by FISH and protein expression by immunofluorescence in breast tumor

Images a-f: PE. g, h, i: 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/pe-Cy5; d: cyclinE/cyclin B1; e: Co-expression of cyclinD1/cyclin E; f. cyclinD1/cyclin B1; Bar: a-f: 10 µm; magnification: g- i: 1000x.[65].

The mosaic phases and protein expression of CD44/CD24 in a breast cancer patient. The mosaic phases and protein expression of CD44/CD24 in a breast cancer patient is provided in Figure 11.

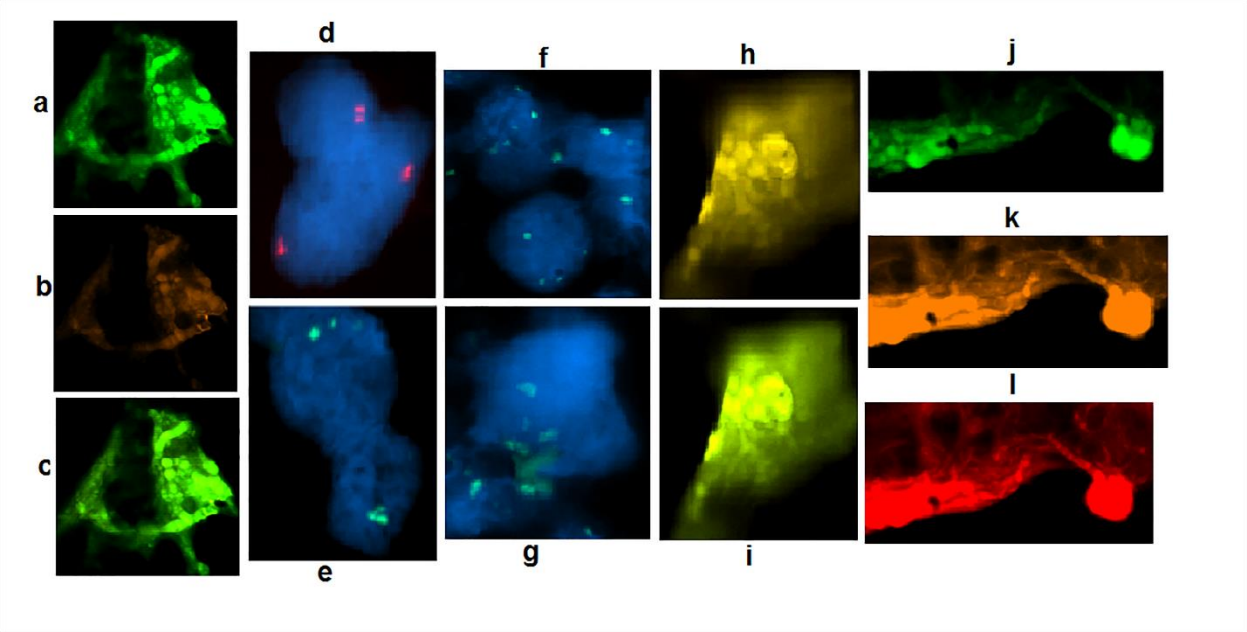


Figure 11. Mosaic phases and protein expression of CD44/CD24 in a breast cancer patient
j-l, Total harmonic PE in cyclin B1 & CDC25A (k,l); Cen: centromere
a,b,c: CD44+, CD24- & merged image -: +stem cell (Scale Bar: 10 µm).d-g: 3cen-aneuploidy in G2, G1 (d); 1cen aneuploidy in G1 (e, f ; magnification: 1000x); g: MPs as G1/S/G2. h & i: Cyclins E/B-, & cyclin E/PCNA co-expression (Scale Bar: 20 µm). j: presents expression of cyclin E, and in J-l: total harmony for PE in cyclin B1 & CDC25A (k,l) is remarkable.
Cen: centromere [65].

Conclusion

The following unmasked definitions may clarify the provided evolutionary manner:
1) Mosaic cellular population; 2) polymorphism;
3) Confused cell cycle as paving the way towards clinical managements and classifying cancer therapeutic protocole by targeting the CDKs inhibitors.

Referring to the cellular destiny, the strategic fate of cell cycle is based on the diverse MPs, i.e., , backward and forward processes, which is partly related to the molecular and functional mechanisms. And of course the environmental factors, including nutrition has an influential impact as well.

Nature programmed to dictate the therapeutic channels through the cell cycle in cancer patients. This is '***an evolutionary forward and backward cell cycling***' [65].

Abbreviations: 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.

Section 6. CANCER THERAPY

To define definite molecular and functional characteristics of an apparently normal case- is an architected ability, because there is no evidence of an original cellular characteristics.

Telomerase, an enzyme in the cell nucleus, is intricately involved in the cancer process through interactions with telomeres, the protective structures at the ends of chromosomes. In most normal human cells, the action of telomerase is repressed and subsequently telomeres, progressively, shorten within each cell division. In contrast, most human

tumors utilize telomerase, resulting in the stabilized telomere length.

By translating the Science to Clinics, and due to the expectation of the clinicians from tumor biology and their apprehension about the cancer therapy, the selected information's are summarized:

1) The biological frames in the Triple-negative breast cancer (TNBC) include heterogeneous-behaviors, prone to metastases with poor prognosis [66].

2) Inhibition of ATM was the fundamental aim for sanitizing the cancer cells in contradiction of genotoxic stress to modulate ATM behavior in cancer therapeutic strategy [67].

*F*urther role of epigenetic and/or post-transcriptional machinery is required to be considered. Besides, other inhibitors including PARP, CHK1/2, WEE1, and DNA-PKcs are also under the clinical advance to facilitate the therapeutic developmental process of ATM and ATR inhibitors [68].

By considering the impact of PE on the clinical management, the manner of cross-talk between ATM and other involved proteins is required to be characterized. However, the clinical correlations in astrocytoma and meningioma are summarized:

1-Low Grade Astrocytoma and meningioma are correlated with PE including Cyclin D2/P53/ Rb1/ ATM.

2- Up-regulated ATM in response to DNA damage reflecting high ATM- PE, by playing role in high grade BTs.

3-High PE in ATM/P53 indicates benign meningioma.

4-Low PE in ATM/Rb reflects negative harmonic expression in astrocytoma.

5-High PE of CDC25A is traced in glioblastoma.

In spite of identification of five more susceptibility loci in breast cancer [69], an everlasting resistance within the cell cycle exists; and the routine chemotherapy is applying [70].

In 2007, It was reported that CDK1 is capable to promote the mammalian cell cycle [71]. However, the application of either classic cytogenetics and/or modern molecular cytogenetics by fluorescence in situ hybridization (FISH) play an exceptional role in unmasking the specific chromosomal alteration [72,73]. Later, the procedure followed by unveiling the 'Mosaic phases' (MPs) as an innovative cell cycle evolutionary event with an influential role of chromosomal behavior in cancer- diagnosis and therapy at single cell level [65].

In mammalian, cell cycle is systematized by the cyclin-dependent kinases (CDKs), which is modulated by numerous cyclins as the activators and inhibitors. The deregulation of CDKs in cancer cells is supported by the genetic/ epigenetic alterations in CDKs, through the mitogenic and regulators or upstream pathways [74,75].It was reported that CDK2, CDK4 and CDK6 are only required for the proliferation of specific cell types. In addition, CDK1 is decisive for cell division in the embryo. Furthermore, CDK1 is necessary for motivating the cell cycle in all cell types, at least until mid-gestation.

The highlighted points include:

1) Although the CDK1 is required for the cell cycle, but the aim of CDKs in interphase is proliferation of particular cells;

2) they have also referred to the requirement of tumor cells for proliferation by relying on the specific interphase CDKs for proliferation; and

3) they have highlighted the selective inhibition of CDK, may have the therapeutic impact on the selected human cancer (s), in addition, abnormal regulation of CDK leads to the spontaneous proliferation, chromosomal alterations and genomic alterations at genomic unpredictability.

Furthermore, Malumbers and Barbacid in 2005 proposed that tumor cells may, selectively, have choice for each CDKs which is essential to be considered for the CDK inhibition based therapy [76].In fact it may be the highlighted point for personalized therapy. For instance, CDK4 is, required for the developing the mammary gland tumors through the influence of the package of *Erbb2*, *Hras* or *Myc* set [77].

As an everlasting cell biological model, CDKs have crucial and dynamic role for circulating separately cell cycle phase, so therapeutic methods that block CDK activity, are doubtfuly capable to hit tumor **cells** [77].

In brief, backing to the initial statements by van den Heuvel in 1993 [78]: 1) CDKs support impulsive proliferation, genomic instability and chromosomal behaviors in cancer cells, 2) by imposing persuasive role through the cell cycle; and 3) the DNA damaging process and mitotic barriers are encouraging tumor cell cycles. But, the insight on cancer has been gradually reformed.

The fate of cell cycle within the junction of tumorigenesis and therapeutic frame has been previously reviewed[79] and the following events within the cell cycle territory are, partly, summarized:

- Regulation of metabolism in cancer cases occurs at the post- translational stage.

- Cancer metabolism occurs: 1) through phosphorylation of metabolic enzymes, 2) or by CDKs.
- CDK inhibitors restore cancer immune tailing by blocking the immune checkpoint.
- Missreugelated progression in cell cycle machinery is considered as the sign of tumorigenic process.

It is also stated that therapies targetting cell cycle mechanism not only repress the division of cancer cells; in addition, reverse cancer metabolism and restore cancer immune surveillance.

By highlighting the past, cellular proliferation requires maintaining the telomeres. Thus, cancer cells turn on genes responsible for telomerase production which in humans normally ceases after birth. Suppressing telomerase is an obvious target for the development of anticancer therapies [80]. At the same period, Weinberg has stated "Creation of human tumour cells with defined genetic elements" [81]. Two years later, Pendino and the colleagues focused on the less complicated malignant tissue, i.e., "leukemia cell differentiation" in which they have highlighted the Retinoids role in "down-regulating telomerase and telomere length in a pathway distinct from leukemic 7 cell differentiation" **82.**

Application of Retinoids include two pathways to influence telomerase activity; i.e., downregulating hTRT, telomerase suppression gradually within two weeks of retinoic acid therapy, subsequently by telomere shortening, growth arrest, and cell death. Telomerase expression is an efficient and selective target of retinoids in the tumors therapy **82.** But, Retinoids are not capable alone to inhibit telomerase. Six years later, Otto and Sicinski reported the key role of cell cycle proteins **83.**

Let's consider the guardian therapeutic strategy by considering the fundamental territories at molecular, functional, and both global and single cell level. Destinations of such journey includes 1)Telomeres/Telomerase; 2)Sub-telomeres; 3) Epigenetics/Methylation; 4-6) Hit-episodes; 7)Cell cycle-phases (TTStEMCC-Ph), and 8)chromosomes. The sub-destination considers the Genomic/Somatic Ratio to create the evolutionary based platform [65]. The cell cycle-based strategy requires a multi-panel experimental exploration by application of traditional and molecular cytogenetics assays, based on very high enumeration at single cell level. The outcomes provide the personalized therapeutic protocole based on ' TTStEMCC-Ph' coding system. The code no. 6 provides the cell cycle secret strategy through the entire cell cycle phase, by passing through the barriers, and /or the backward direction. Such phenomena is the Natural intelligence (NI) which is capable to direct the therapeutic policy, accompanied by the fundamental results of destinations 1-5.

It's worth to highlight the influential status of the involved stem cells, as the example including CD44/CD24 (**Figure 12**) in breast cancer; CD133 (**Figure 13**) in neural neoplasms; and the pedigree-based platform by considering the preventive, predictive and early detection of the probands' relatives who may be predisposed to the neoplastic disorders including cancer.

The benefit of the provided image 13 is for follow-up study and tracing the probable migration of the invasive cells, as the metastatic circulating tumor cells from the breast, through the blood stream into the brain, as early as possible (**Figure 13**).

Furthermore, by highlighting, the applicability, safe and availability of dental pulp samples from the family members, the following points are conveyed: In breast cancer, the capability of specific stem cells package, i.e., CD44+/CD24- is reflective of the influential value of the stem cell for diagnostic aim in breast cancer. Furthermore, an early detection of stem cells' status is now facilitated through the non-invasive circulated breast cells or any other involved organ at blood stream, or a touch of buccal smear, as frequent as required (Personal archive)

The last and not least, micro-and macro-environmental factors are the top consideration for the parents, the embryonic-, fetal stage, and post-birth periods. The human's life is a real circulating rounds, but not the cell cycle with combination of forward and backward circling, which fate the health and malady. The major research aim is translating the applicable achievements, based on the non-invasive approach to the cancer patients and their targeted relatives through their pedigrees as prompt as possible.

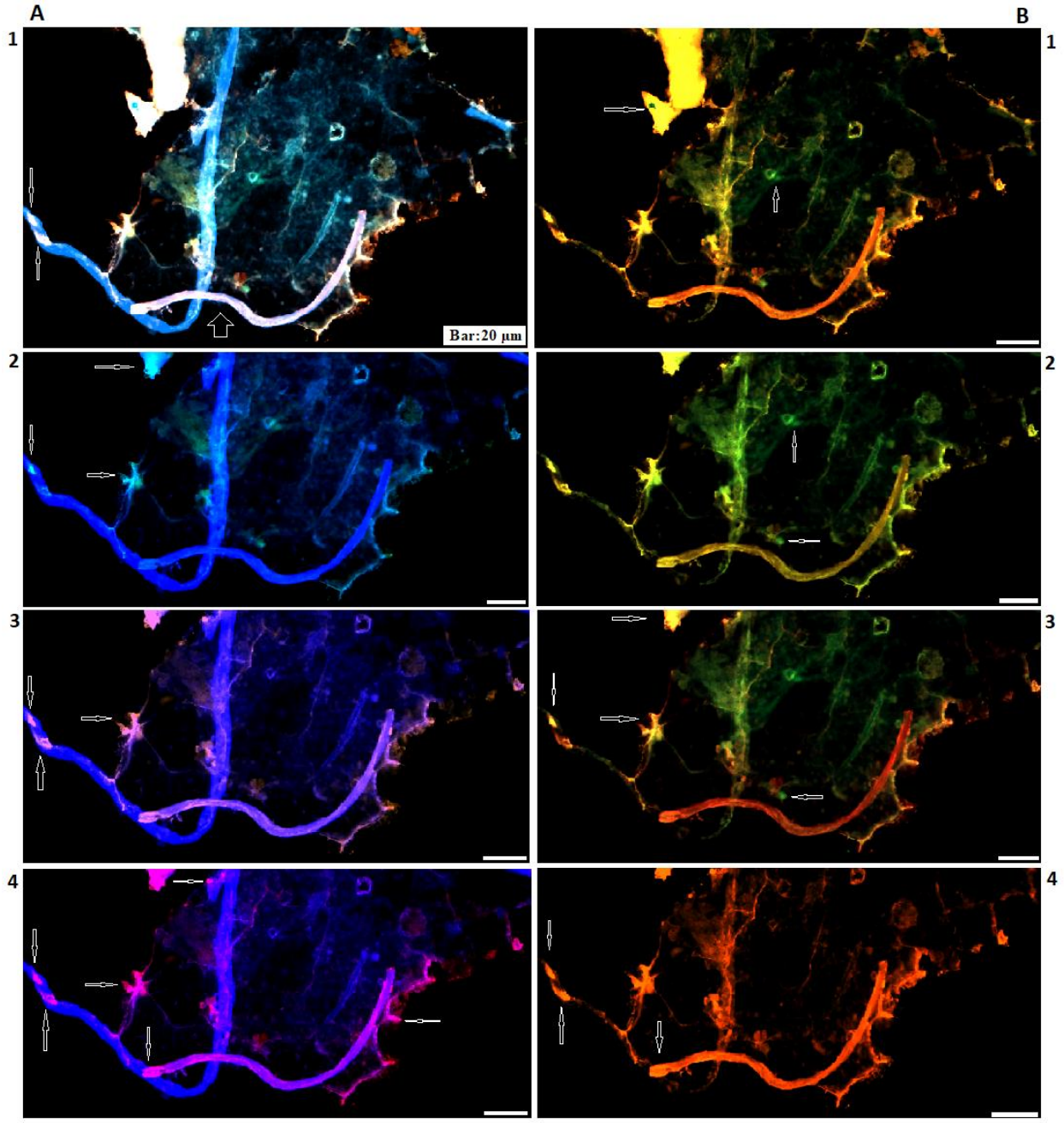


Figure 12. Protein expression of breast tumor of a patient affected with primary breast cancer.
Stem cells including CD44, CD24, and ATM are respectively conjugated with FITC (green), Pe-Cy5 (red), and R-pe (orange),. Status of CD44+/CD24- is reflective of positive stem cells, as shown in only 2-3 cells in this image(B-2).
A1: Dapi-CD44/CD24/ATM; A2: Dapi/CD44; A3:Dapi/CD24; A4:Dapi/ATM
B1: CD44/CD24/ATM; B2: CD44/CD24; B3:CD44/ATM; B4: CD24/ATM

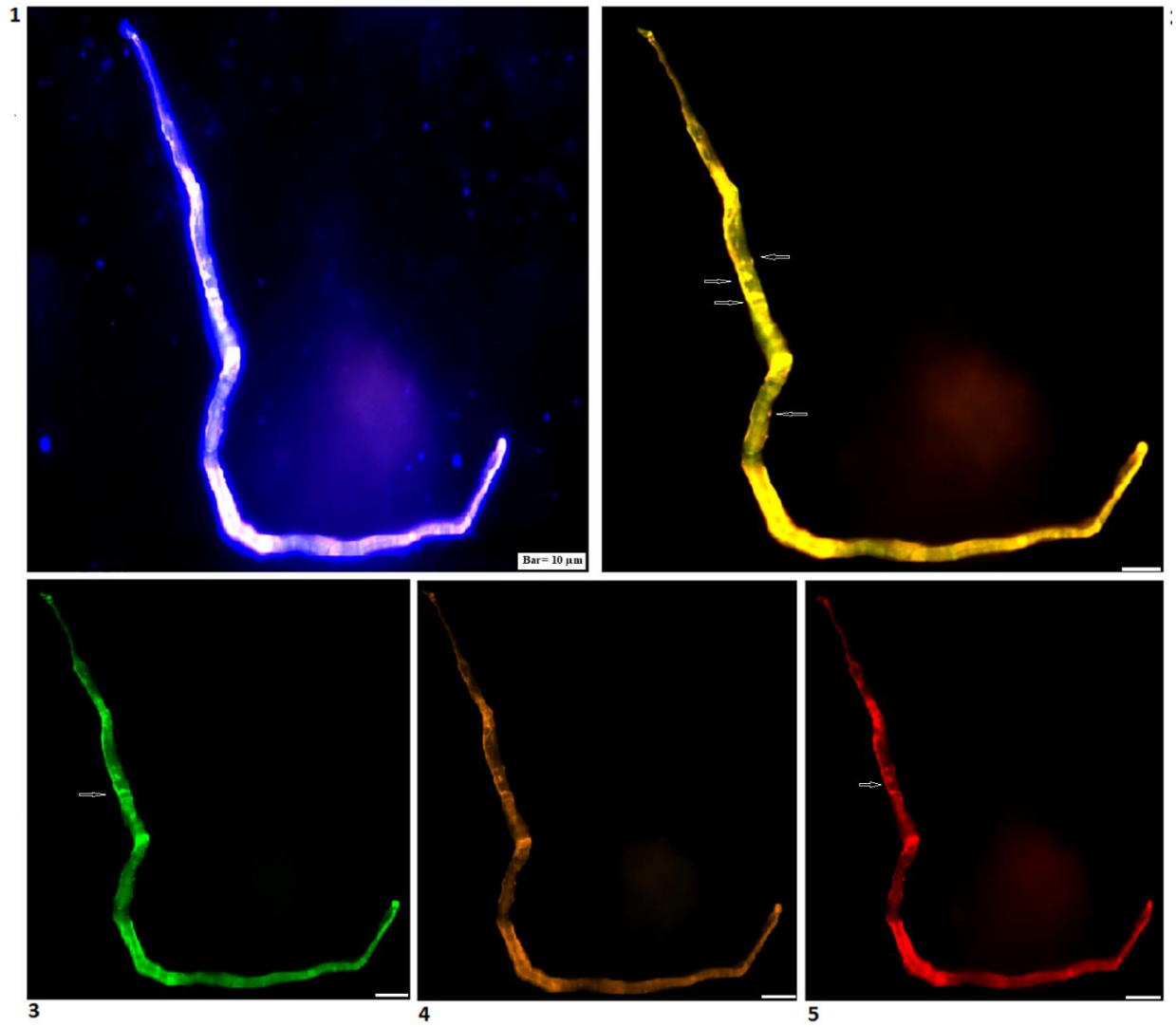


Figure 13. Protein expression of CD133, VEGF and EGF in the circulating tumor cells of a patient affected with primary breast cancer.

circulating tumor cells in a patient affected with breast cancer:

1) Cells conjugated with Dapi/FITC/Rpe/ Pe-cy5; 2) same cells conjugated with FITC/Rpe/ Pe-cy5; 3) cells conjugated with FITC/ CD133: 4) cells conjugated with Rpe/VEGF; and 4) same cells conjugated with Pe-cy5/EGF CD133: neural stem cells; VEGF: vascular endothelial growth factor; and EGF: epidermal growth factor.

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