Review Article

CONTRIBUTIONS OF CYTOGENETICS TO CANCER RESEARCH

CONTRIBUIÇÕES DA CITOGENÉTICA EM PESQUISAS SOBRE O CÂNCER

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ABSTRACT: The two conflicting visions of tumorigenesis that are widely discussed are the gene-mutation hypothesis and the aneuploidy hypothesis. In this review we will summarize the contributions of cytogenetics in the study of cancer cells and propose a hypothetical model to explain the influence of cytogenetic events in carcinogenesis, emphasizing the role of aneuploidy. The gene mutation hypothesis states that gene-specific mutations occur and that they maintain the altered phenotype of the tumor cells, and the aneuploidy hypothesis states that aneuploidy is necessary and sufficient for the initiation and progression of malignant transformation. Aneuploidy is a hallmark of cancer and plays an important role in tumorigenesis and tumor progression. Aneuploid cells might be derived from polyploid cells, which can arise spontaneously or are induced by environmental agents or chemical compounds, and the genetic instability observed in polyploid cells leads to chromosomal losses or rearrangements, resulting in variable aberrant karyotypes. Because of the large amount of evidence indicating that the correct chromosomal balance is crucial to cancer development, cytogenetic techniques are important tools for both basic research, such as elucidating carcinogenesis, and applied research, such as diagnosis, prognosis and selection of treatment. The combination of classic cytogenetics, molecular cytogenetics and molecular genetics is essential and can generate a vast amount of data, enhancing our knowledge of cancer biology and improving treatment of this disease.

KEYWORDS: Tumor. Chromosomes. Chromosomal Instability. Cytogenetic Techniques.

INTRODUCTION

Cytogenetics is a branch of genetics that focuses on chromosome studies, a subject that has always been one of the most exciting areas of cytology. Cytogenetics is at the interface of genetics and cytology, and it employs unique tools that enable entire genome of a eukaryote to be viewed in the form of condensed blocks of genetic material (GUERRA, 2002). The origin of human cytogenetics is attributed to Walther Flemming, who published the first illustrations of human chromosomes in 1882 and used the term mitosis for the first time. In 1888, Heinrich Wilhelm Gottfried von Waldeyer-Hartz first used the term chromosome when referring to the colored bodies observed during mitosis. Walter Stanborough Sutton and Theodor Boveri independently developed the chromosome theory of inheritance in 1902 and Sutton was responsible for the union of cytology and genetics, referring to the study of chromosomes as cytogenetics. The correct determination of the human diploid chromosome number as 46 by Joe Hin Tjio and Albert Levan occurred in January 1956 at the University of Lund, Sweden (TIJO; LEVAN, 1956; GERSEN; KEAGLE, 2005).

Since its origin, cytogenetics has been used in many fields such as taxonomy, plant breeding and clinical analysis, including cancer diagnosis and prognosis. With the advancement of molecular techniques, classic cytogenetics has been somewhat forgotten over the years, but recent studies have shown the importance of both, classic and molecular cytogenetics, as tools to understand particular aspects of tumor biology.

Genetic instability and cancer

Cancer results from genetic alterations that occur in a single somatic cell. All of the cells generated by the first neoplastic clone accumulate a series of genetic and epigenetic alterations that change gene activities and modify the cellular phenotype through a selection process (PONDER, 2001). Genetic instability or changes in the chromosome number or structure are important factors in oncogenesis. The consequences of genetic instability include changes in the copy number of one or more genes and changes in gene expression or gene structure, some of which may modify the corresponding protein structure. These genetic alterations can increase or decrease the protein's activity or create a different protein with a new function (SAUNDERS et al., 2000). The most important genetic alterations in tumor cells occur in the genes responsible for controlling cellular proliferation (proto-oncogenes and tumor suppressors), resulting in the uncontrolled growth that is characteristic of cancer. Tumor suppressor

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genes perform diverse cellular functions generally related to the control of cellular proliferation, but these genes are inactive in tumor cells, while oncogenes with activator mutations stimulate cell growth (OJOPI; NETO, 2002).

When neoplastic cells mreplicate into a unique cellular mass, the tumor is considered benign. A tumor is considered cancerous only if it presents the characteristics of malignancy, such as the ability to escape the initial mass via blood or lymphatic vessels, invade the neighboring tissues and generate secondary tumors or metastases (ALBERTS et al., 2010). According to Hanahan and Weinberg (2000), malignancy depends on the acquisition of certain characteristics. It was initially proposed that the transformation of normal cells into malignant cells depends on six mutations that provide insensitivity to anti-growth signals, sustain angiogenesis, prevent apoptosis, provide self-sufficiency regarding growth signals, invoke the potential for limitless replication and allow tissue invasion and metastasis.

Additional hallmarks of cancer include the evasion of immune surveillance (KROEMER et al., 2008), damaged DNA and several causative conditions of cellular stress such as DNA replication and mitosis as well as oxidative proteotoxic and metabolic processes (LUO et al., 2009). Negrini et al. (2010) suggested the inclusion of genomic instability as one of the hallmarks of cancer because it is observed at all stages of cancer. Hanahan and Weinberg (2011) revisited the hallmarks of cancer and proposed a next set of characteristics, initially including deregulation of cellular energetic processes and the ability to avoid immune destruction as emergent characteristics, and the promotion of inflammation and genome instability and mutation as enabling characteristics.

An important class of genome instability is chromosomal instability, generally designated as CIN. The term CIN is applied when cancer cells present an instable karyotype that generates a heterogeneous population of cells, which is a common feature of many tumors. Although CIN can drive aneuploidy, not all aneuploid cells exhibit CIN, as CIN has been observed in aneuploid cells with a very stable cytotype (DAVID *et al*., 2012). The presence of chromosomal abnormalities is ubiquitous in solid tumors; therefore, cytogenetic analysis is very important to improve our understanding of the role of karyotypical abnormalities generated by CIN in cancer development and progression.

How can cytogenetics contribute to cancer research?

Cytogenetics is important to understanding carcinogenesis because it is closely linked to mutations and changes in chromosomal structure. The genetic alterations observed in malignant cells that are associated with the initiation or proliferation of a tumor may be mediated by large chromosomal changes and thus may be cytogenetically visible. The cytogenetic rearrangements in tumors are divided into three categories based on the mechanism by which they promote tumor growth (Table 1) (CASARTELLI, 1993).

Table 1. Types of cytogenetic rearrangements found in tumors and the influence of these aberrations on tumor progression (CASARTELLI, 1993).

Cytogenetic rearrangements	Mechanism by which it promotes the growth of the tumor
Translocations, inversions and insertions	Affect genes at a limited distance from the point of breakage and can result in the chromosomal deregulation of genes or formation of chimeric oncogenes.
Chromosomal deletions and monosomies	Demonstrate that the loss of function of some genes is important for the initiation or progression of a tumor.
Polisomies, amplifications, isochromosomes, extrachromosomes, microchromosomes, and double-minutes, among other chromosome markers	Can alter the expression of hundreds or thousands of genes, with the physiological effects varying depending on the dosage of genes with altered expression.

The cytogenetic data indicated that the chromosomal changes in a tumor could be used for tumor classification, diagnosis and prognosis. Sometimes the histological features of a tumor overlap with the characteristics of other tumor types, making it almost impossible to differentiate among them using histological methods. For example, it is difficult to differentiate myxoid liposarcoma from other types of liposarcoma. The discovery of the translocation between chromosomes 12 and 16 facilitated the diagnosis of this type of sarcoma. Some types of leukemia, particularly the acute type, are characterized by specific chromosomal events, enabling their differentiation into different subtypes (CASARTELLI, 1993; HAHN; FLETCHER, 2005). Several studies have demonstrated the importance of gene fusions that result from chromosomal translocations in cancer progression. These translocations juxtapose portions of two genes, creating chimeric gene products with a different and specific role in cell proliferation and changing the expression of the genes involved (BARR, 1998).Such chromosome alterations have been identified in leukemias, lymphomas and sarcomas; one example is the translocation between chromosomes 12 and 15 observed in congenital fibro sarcoma which generates a marker chromosomes with the fusion of the ETV6 and NTRK3 oncogenes (Figure 1). Another examples are the translocations observed in alveolar soft tissue sarcomas and chronic myeloid leukemia (CML). In alveolar soft tissue sarcomas, the translocation between chromosomes X and 17 causes the fusion of the *ASPL* and *TFE3* oncogenes, is present in more than 90% of tumor cells and has strong diagnostic utility. In CML, a translocation between chromosomes 9 and 22 generates a marker called Philadelphia chromosome, which presents a fusion between BCR-ABL1 oncogenes (GERSEN; KEAGLE, 2005).

According to the Catalogue of Somatic Mutations in Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/), 9014 gene fusions related to human cancer had been described until June 2013. This large number is additional evidence of the essential role of chromosomal rearrangements in the progression of cancer.

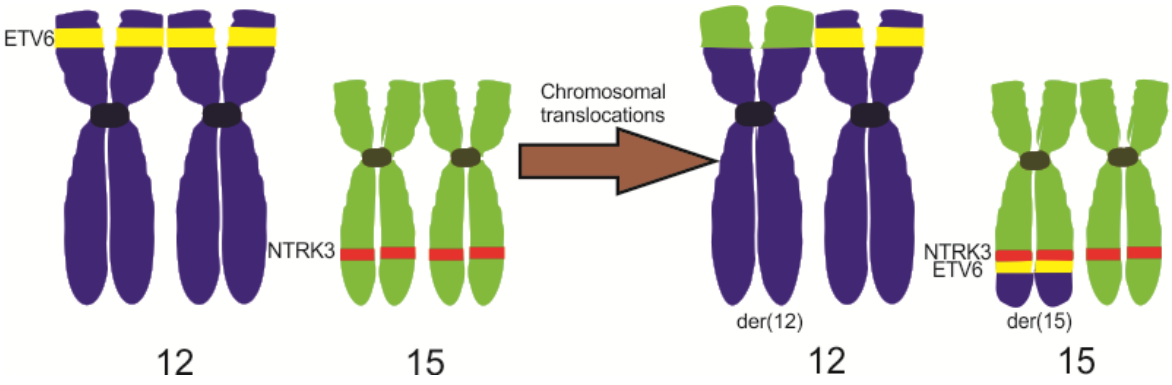

Figure 1. Schematic representation of the translocation between chromosomes 12 and 15. (adapted from GERSEN; KEAGLE, 2005).

The gene fusions mentioned thus far are associated with specific tumor types; therefore, cytogenetic studies of chromosomal rearrangements can be a useful tool for identifying specific tumors. For example, cytogenetic analysis is imperative for CML patients because in addition to establishing the diagnosis, it can predict the clinical transformation from the chronic phase to the accelerated phase or blast crisis. Cytogenetic analysis is used in CML patients to identify the fusion between the oncogenes BCR and ABL1, and the prescription of certain drugs, such as imatinib mesylate (STI571, Gleevec™), is based on cytogenetic results (GERSEN; KEAGLE, 2005). Different genetic subtypes of multiple myeloma (MM) have been identified, which present different underlying biologic features and heterogeneic clinical outcomes. In MM, classic cytogenetics and FISH permit the identification of high-risk genetic features and allow patients to be stratified into a high-risk group or a group with a better prognosis (SAWYER, 2011).

The data mentioned above indicate that cytogenetics is useful for tumor classification and for evaluation of its invasiveness. Since its origin, discoveries in cytogenetics have been applied to

cancer and other genetic diseases. A significant challenge for future research will be to integrate data from conventional cytogenetics, fluorescence in situ hybridization (FISH), interphasic fluorescence in situ hybridization (I-FISH), gene expression profiling (GEP), comparative genomic hybridization (CGH), array comparative genomic hybridization (aCGH), and single-nucleotide polymorphism (SNP) arrays into improved diagnostic and prognostic tools to guide cancer therapy (SAWYER, 2011). Below, some classic and new cytogenetic techniques that have been applied to cancer research to better understand genetic and chromosome instability are described.

Cytogenetic techniques applied to the study of cancer

Classic cytogenetics

Cytogenetics has an essential role in the detection of human diseases, considering that numerical abnormalities, such as aneuploidy, and structural abnormalities, such as translocations, inversions, deletions and duplications, are easily observed using classic protocols. The first advance in cytogenetics was the application of colchicine and hypotonic solutions for cell treatment, which represents a milestone in cytogenetic studies (GERSEN; KEAGLE, 2005). Treatment with these solutions improved the quality of the mitotic chromosomes and consequently chromosomal analysis. Colchicine is an alkaloid derived from the plant *Colchicum autumnale* that binds to tubulin dimers, preventing microtubule polymerization. In addition to its effect on polymerization, colchicine disrupts tubulin dimers, preventing the formation of the mitotic spindle and consequently the segregation of sister chromatids (WILSON; MEZA, 1973).

Treating biological materials with hypotonic salt solutions is an important step in obtaining high-quality metaphase chromosomes, a prerequisite for the subsequent cytogenetic analyses. Exposing metaphase cells to a hypotonic solution disperses the chromosomes throughout the cytoplasm once they leave the central region (CLAUSSEN et al. 2002).

One way to visualize structural karyotypic alterations is by producing chromosomal markers, called banding. Among the procedures for banding, G-banding is the most widely used method for recognizing pairs of chromosome. Giemsa banding occurs from the interactions of DNA and protein with the thiazine and eosin components of the stain; the technique tends to denature AT-rich DNA (BICKMORE, 2001). The AT-rich DNA is late replicating and corresponds to the heterochromatic regions of the chromosomes that contain relatively few active genes. In contrast, the CG-rich DNA, which appears as light bands, are the early replicating euchromatic regions. The G-bands also correspond to the condensed chromomeres of meiotic chromosomes (GERSEN; KEAGLE, 2005)

G-banding has been effectively and widely used in oncology research and clinical analysis. For example, when G-banding was performed on metaphase tumor cells from patients with congenital fibrosarcoma, it revealed trisomy of chromosome 15 and the translocation between chromosomes 12 and 15 as a structural abnormality (SANDBERG, MELONI-EHRIG, 2010).

According to Buwe et al. (2003), neoplastic cells often exhibit complex chromosomal aberrations in many different marker chromosomes. The less pronounced banding patterns of certain chromosomes make it difficult to assign genetic abnormalities to them using conventional banding techniques. One important event in cytogenetics was the cooperation between classic cytogenetics and molecular biology to create molecular cytogenetic techniques that involve the manipulation of genetic material. Molecular cytogenetics began with *in situ* hybridization, another milestone in the evolution of cytogenetics, as described by Pardue and Gall in 1969.

The continuous advancement of molecular cytogenetics included the development of fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH) and spectral karyotyping (SKY). These techniques are excellent tools for genetic analysis. The banding patterns obtained using molecular cytogenetics are the focus of any clinical cytogenetics laboratory because technical difficulties such as low-quality chromosomes or scarce tumor cells are overcome more easily with the help of these advanced cytogenetic techniques.

Fluorescence In Situ Hybridization (FISH)

FISH is a useful technique for the detection of chromosome abnormalities because it enables analysis of interphase cells (I-FISH), characterization of marker chromosomes, screening a large number of cells within a short period of time and the ability to study samples with few or poorly assessable metaphase chromosomes (OUDAT et al., 2001).

FISH can be used for mapping specific chromosomal loci and detecting numerical and structural cytogenetic aberrations. FISH allows the visualization of the genomic target using metaphase

chromosomes, interphasic nuclei or tissue sections. Its application is particularly important for detecting translocations, inversions, insertions and microdeletions, as well as identifying marker chromosome and characterizing chromosome break points (LE SCOUARNEC; GRIBBLE, 2012). FISH is considered the best method to detect v-myc (myelocytomatosis viral oncogene, also called MYCN) in neuroblastoma where a translocation between chromosomes 8 and 14 causes its uncontrolled expression, as well in other tumors, such as medulloblastoma, rhabdomyosarcoma and Wilms tumor, in which this gene is amplified. In hematological cancers such as CML, the loss of DNA at the translocation break points can be observed using FISH. Because of its sensitivity, this technique should be considered for monitoring cancer after treatment. The FISH procedure is a sensitive, fast and indispensable complement to conventional cytogenetic techniques (WAN; MA, 2012).

Despite their efficacy in detecting several chromosomal rearrangements, FISH or chromosome banding alone does not comprehensively characterize a disturbingly large number of chromosomal aberrations (GARINI et al., 1996). One important advance in the FISH technique is spectral karyotyping (SKY), an imaging technique for the analysis of FISH experiments. The SKY technique allows easy visual interpretation of FISH results. It is based on the simultaneous hybridization of 24 combinatorially labeled human chromosome painting probes, and visualizing all of the human chromosomes in different colors is achieved by spectral imaging. This technique can be used to characterize translocations involving non-homologous chromosomes; however, this procedure does not allow the detection of structural abnormalities such as inversion, deletion and duplication on the same chromosome because each chromosome has a unique color (GARINI et al., 1996; WAN; MA, 2012).

According to Bayani and Squire (2002), SKY has been used to identify various tumor groups. These include hematological malignancies, sarcomas, carcinomas and brain tumors. The intent of SKY is to identify specific chromosomal abnormalities that may provide insight into the genes involved in the disease process as well as to identify recurrent cytogenetic markers for clinical diagnosis and prognostic assessment. Rare tumors such as alveolar soft tissue sarcomas have also been studied using SKY. In addition to cytogenetics, Holland et al. (2012) used other molecular genetic techniques. The authors emphasized the necessity of combining complementary methods to obtain comprehensive information and understand tumorigenic aberrations.

Comparative Genomic Hybridization (CGH)

Another complementary technique used with classic cytogenetics is CGH. This technique detects unbalanced chromosomal changes (loss/gain) using a small amount of DNA. In CGH, normal DNA (called reference DNA) and tumor DNA are labeled with different fluorochromes, then both DNAs are hybridized to normal human metaphase chromosomes, and fluorescence ratios along the length of the chromosomes provide a cytogenetic representation of the relative DNA copy-number variation. If the chromosome or chromosomal sub-regions have the same copy number as the target DNA sample, the fluorescence observed has equal contributions from the tumor DNA and the normal DNA, which is the case for soft tissue sarcomas. If there are deletions in the tumor sample, the labeled region will have the color of the reference sample. In cases of gains, the tumor DNA fluorescence is predominant (PINKEL et al., 1998). The advantage of CGH is that only the tumor DNA is required for the molecular cytogenetic analysis, and tumor DNA can be easily obtained by micro dissection of tumor samples, a feature that made CGH one of the most commonly used techniques in research and molecular diagnostics (WAN; MA, 2012).

According to Wada *et al.* (2002), the chromosomal aberrations revealed by CGH are common in both Hürthle cell carcinomas and adenomas, and the detected chromosomal gains may be predictive for the recurrence of the disease. A CGH study of a patient with a rare bone tumor called malignant triton tumor (MTT) revealed amplification in several chromosomes (1, 6, 16, 17, 19, 20 and 22), consistent with a report describing recurrent genomic aberrations in chromosomes 1, 16, 17, 19 and 22, suggesting the involvement of several oncogenes in the genesis of MTT (KOUTSIMPELAS et al., 2011). Yeh et al. (2012) presented a case that highlights the use of two methods that help for diagnosing spindle cell melanoma, the CD34 fingerprint technique and CGH. In this study, a shave biopsy from the cheek of a 58-year-old man demonstrated a thin invasive melanoma, and CGH demonstrated a gain in the long arm of chromosome 6, a loss in the short arm of the same chromosome and a gain in chromosome 7, supporting the diagnosis of spindle cell melanoma.

Array-based CGH (aCGH)

An adaptation of CGH is array-based CGH (aCGH). According to Harvell et al. (2004), aCGH is a method that combines traditional CGH and microarray technology. With this technique, tumor and normal genomic DNAs are differentially fluorescently labeled and co-hybridized onto an array containing mapped DNA sequences, providing measurements of tumor copy number changes at high resolution across the genome. In their study, Harvell et al. (2004) used aCGH as a diagnostic test to distinguish between Spitz nevus and melanoma using the DNA isolated from formalin-fixed and paraffin-embedded samples, demonstrating the effectiveness of this method as a diagnostic tool to differentiate Spitz nevus from melanoma.

Cytogenetic events related to cancer progression

Polyploidy and Cancer

The number and structure of the chromosomes in many cancer cells is highly variable, a condition known as aneuploidy and that is a consequence of an initial polyploidy. Aneuploidy is the situation where the number of chromosomes is not an exact multiple of the characteristic haploid number for the species. Aneuploidy is often observed in tumor cells, primarily in solid tumors. The correlation between cancer and aneuploidy has been known for decades; however, the answer to this central question is still unknown: is aneuploidy a contributing cause or merely a secondary consequence of malignant transformation? (LENGAUER et al., 1997; STORCHOVA; PELLMAN, 2004).

Eukaryotic organisms generally have a diploid number of chromosomes. The diploid state is preferred and evolutionarily maintained. It allows for sexual reproduction and facilitates genetic recombination. However, there are a surprising number of exceptions, especially in somatic cells.For example, cytokinesis failure process produces differentiated binucleated tetraploid progenies in liver cells (GENTRICA et al., 2012). There are organisms that have more than one diploid chromosomal complement, as observed by Morelli *et al.* (1983) in a fish specimen. Moreover, the chromosomal complement may differ within the same organism, depending on the cell type, and an increased number of chromosomes is widely observed in tumor cells (STORCHOVA; PELLMAN, 2004).

There are two different classes of polyploidy, allopolyploidy, in which two or more related but not identical genomes are combined and autopolyploidy, in which two or more identical genomes are combined. Polyploid cells can be formed by three different mechanisms: an abortive cell cycle, cell fusion and endoreduplication (STORCHOVA; PELLMAN, 2004).

According to Larizza and Schirrmacher (1984), tumor cells that have metastatic properties often have a higher gene dosage than that of the original cells. This fact is demonstrated by an increased ploidy level, chromosomal duplication and gene amplification. The acquisition of the large number of chromosomes observed in tumor cells may be the result of endoreduplication or somatic hybridization (cell fusion). In some cell types, cell fusion is a part of normal development, producing terminally differentiated cells such as muscle cells and osteoclasts. Cell fusion causes an intracellular disorder with changes in the genetic structure and consequent instability. This occurrence can cause the emergence of aneuploid clones. These clones may have neoplastic characteristics and have an unstable or relatively stable chromosomal complement, ranging from triploid to tetraploid (HESELMEYER, 1997; STORCHOVA; PELLMAN, 2004; DUELLI; LAZEBNIK, 2007).

The endoreduplication observed in many tumor cell lines is a common event in arthropods and is well characterized in *Drosophila* salivary glands (forming polytene chromosomes) and in megakaryocytes, which are the mammalian cells responsible for platelet formation (STORCHOVA; PELLMAN, 2004). The process of endoreduplication results in diplochromosomes consisting of four chromatids, instead of two, grouped side by side. Endoreduplication occurs when the cells proceed through two rounds of DNA replication without chromatid separation (SUMNER, 1998).

Endoreduplication occurs when certain mechanisms that drive the sequential progression of the G1, S, G2 and mitotic phase (M) of the cell cycle are modified. Usually the chromosomes replicate only once per cell cycle and mitotic progression (M phase) is required for the release of other points of replication origin, initiating the next round of chromosomal duplication (LARKINS et al., 2001). The normal mitotic cycle consists of periods of DNA synthesis (S phase) and chromosome segregation (M phase), preceded by the G1 and G2 intervals, respectively. During the cell cycle, the orderly progression of events that causes chromosomal duplication and separation is governed by cyclin dependent kinases (CDKs). In the normal cell cycle, the progression of the S phase requires a complete M phase, but in the process of endoreduplication, this dependency is turned off,

and chromatin is re-condensed even if does not complete mitosis. These mechanisms are regulated by the concentration of CDKs, and inhibitors of these proteins cause polyploidy (LARKINS et al., 2001).

Aneuploidy: cause or consequence of tumorigenesis?

Two conflicting concepts of tumorigenesis are widely discussed: one that states that gene specific mutations initiate and maintain the altered phenotype of tumor cells and another that states that aneuploidy is necessary and sufficient for the initiation and progression of malignant transformation (DUESBERG et al., 2005; DUESBERG, 2007). Aneuploidy, although observed in cancerous cells nearly a century ago and considered the cause of malignant transformation until the 1960s, was ignored for the past 25 years, primarily because the technology of the time failed to identify specific patterns of chromosomal rearrangements in different types of cancer. However, a growing number of articles have reported aneuploidy as the genetic basis of cancer development (STOCK; BIALY, 2003).

The role of intra-genic punctual mutations in cancer is well established. However, the contribution of massive genomic changes collectively known as aneuploidies is less certain. Relating these subjects, it has been suggested that aneuploidy is required for carcinogenesis in mice and that it plays a role in the development of intra-genic mutations during tumorigenesis. The genomic plasticity provided by aneuploidy could facilitate changes in gene dosage that would promote tumorigenesis and accelerate the accumulation of oncogenes and the loss of tumor suppressor genes. These discoveries stimulated the revision of the basic concepts of cancer pathogenesis and have significant implications for the diagnosis and treatment of the disease (PIHAN; DOXSEY, 2003).

The theories based on genic mutations ("genocentrics") state that cancer is caused by the clonal expansion of cells, which accumulate specific mutations leading to carcinogenesis. While this is occurring, how do the normal cells of the body remain free of mutations? According to Duesberg et al. (2005; 2007) the conventional genetic model and epigenetic events cannot explain the carcinogenic properties listed below, which can be explained by the chromosomal theory of cancer:

1) Most tumors are not heritable and are thus extremely rare in infants and a senile disease. According to the genocentric theory, cancer should be a common disease in infants because they could inherit the mutant genes of the father and mother, accumulating the mutations necessary for carcinogenesis. 2) Carcinogenic non-mutagenic agents may cause cancer. 3) The tumors develop only after years or decades after being initiated by carcinogens. 4) The genocentric theory does not offer an exact correlation between cancer and aneuploidy because omnipresent aneuploidy in cancer was not postulated or even predicted. 5) Pre-neoplastic aneuploidies are found in some tissues. These cells are often observed following exposure to carcinogens. 6) The karyotypic and phenotypic variation observed in tumor cells is much higher than the rate of conventional mutations. 7) Since 1960, several cancer-specific chromosome alterations (not random alterations), also known as aneusomies, have been identified. 8) The tumor cell phenotype is too complex to be explained by the mutational theory, and the mutations observed in malignant cells may be a result of aneuploidy; even the types of tumors observed in heritable syndromes can be generated by aneuploidy. 9) Conventional genetic theory explains tumor evolution through specific mutations and Darwinian selections. However, this model cannot explain the nonselective phenotypes of tumor cells. 10). Several genic mutations have been observed in cancer since 1980, but none of them can be called a cancer-causing gene primarily because the mutations found in cancer are not cancer-specific (DUESBERG *et al.*, 2005; DUESBERG, 2007).

Increase in the Nucleolus Organizer Region (NOR) activity as a proliferation marker

NORs are chromosomal regions in which the ribosomal genes (rDNA) are clustered together, from which are transcribed the ribosomal RNAs - rRNAs. These regions are associated with the nucleoli and are responsible for its reorganization at the end of cell division. A group of specific argyrophilic proteins are associated with active NORs, called the argyrophilic proteins of NOR (Ag-NOR), which can be visualized in the nucleus and chromosomes by light microscopy following silver-nitrate staining (MILLER et al., 1976).

The silver nitrate impregnation technique can be used to visualize activity at the site of the rDNA genes. Silver impregnation does not occur at all rDNA sites; only those that are transcriptionally active or have been active and still have residual proteins associated with the rRNA attached to the condensed rDNA cistrons (SCHWARZACHER et al., 1978).

AgNORs activity is frequently used to measure proliferation potential of several tumor

types because these regions are associated with high protein synthesis and consequently the aggressiveness of a tumor; it is interesting that AgNORs are conserved in malignant cells (KANEKO et al., 1991; ISHIDA et al., 1993; OSHIMA; FORONES, 2001). This cell cycle kinetic information is used to measure the proliferation potential of a tumor, a very important feature for diagnosis and prognosis (KANEKO et al., 1991; ISHIDA et al. 1993; AHMED et al., 2011; DE-MORAES et al., 2012). Some evidences indicates that an increase in AgNOR-reactive proteins results in a larger area being occupied in the AgNOR-positive sites, being discriminated individually as aggregates and not as dots, on account of a more intense nucleolar activity (MELLO et al., 2008).

According to Hanemann et al. (2011), the AgNOR staining technique is a useful diagnostic tool for different types of oral squamous cell carcinoma because the differences in AgNOR numeric values can be identified; therefore, the AgNOR staining techniques can be used in histopathology due to its low cost and ease of performance. Aiming to determine the diagnostic accuracy of AgNOR for brush biopsies taken from suspected oral lesions for the early detection of oral cancer, Rajput *et al.* (2010) concluded that AgNOR staining of brush biopsies is an facile, non-invasive, safe and accurate screening method for the detection of macroscopic suspicious oral cancerous lesions. According to these authors, NOR detection can be used in addition to other routine cytological diagnoses for the early detection of oral cancers. It is important to considerer that normal polyploidy cells, as liver cells, also increases the amount of nucleolar organizer region (AgNOR) positive dots or aggregates, as well as oral mucosa exposed to smoking and alcohol (VIDAL BDE, et al. 1994; PAIVA et al., 2004).

Telomere damage and its influence in carcinogenesis

Telomeres are structures present at the end of linear chromosomes and maintain the integrity of the chromosome. These structures ensure appropriate chromosomal structure and function, maintaining the genetic stability of cells. In mammals, as in all vertebrates, telomeres consist of many kilobases of tandem repeats of the TTAGGG sequence, have specific telomere associated proteins and end in a large duplex T-loop (Figure 2) (GRIFFITH et al., 1999). Among the many proteins associated with telomeres, TRF1 and TRF2 are the important ones. TRF1 regulates the telomere length, and TRF2 maintains the integrity of telomere (KARLSEDER et al., 1999). The length of the TTAGGG repeats varies from one species to another. In human germ line cells, telomeres of between 15 to 20 Kb in length have been observed, whereas the telomeres are much longer in mice (*Mus musculus*), ranging from 30 to 50 Kb. In addition to this inter-species variation, the telomere length may vary within species and individually, depending on the genotype, the cell type examined and the replicative history of the cell. Telomeres are responsible for the control of cell division, so that after a certain number of divisions cells enter the replicative senescence pathway (LEJNINE et al., 1995; BLACKBURN; GREIDER, 1995; FYHRQUIST; SAIJONMAA, 2012; RAMPAZZO et al., 2012).

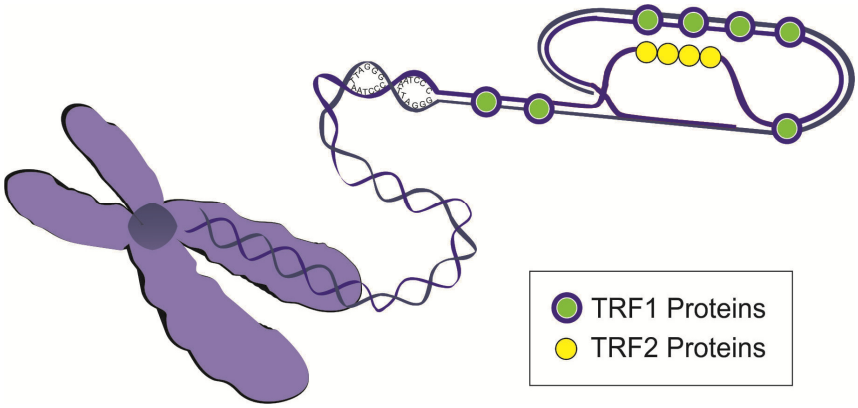

Figure 2. Schematic representation of mammalian telomeres, which are comprised of TTAGGG nucleotide repeats and have many associated proteins, including TRF1 (green) and TRF2 (yellow) (adapted from NEUMANN; REDDE, 2002).

The replication of linear chromosomes presents a challenge because of, the inability of DNA polymerase to complete the synthesis of the end of the linear chromosomes. Because DNA synthesis occurs only in the 5' => 3' direction and requires RNA primers for initiation, the telomeres are not completely replicated by the conventional complex of DNA polymerase. Thus, when the cell divides, the difficulty replicating in the end of chromosome results in shortened telomeres (GILLEY et al., 2005).When the telomeres reach a critical length, they induce the activation of check points very similar to those initiated by damaged DNA. In human cells, short telomeres result in the activation of senescence, and the cells cease replicating (MASER; DEPINHO, 2002).

The progressive shortening of telomeres causes senescence, cell death or genetic instability. Evidence suggests that telomeric shortening contributes to the initiation and progression of malignant tumors in several ways. The genetic instability caused by telomere dysfunction is one of the main factors that predispose cells to malignant transformation (CAMPISI et al., 2001). According to Martinez-delgado et al., (2012) a shorter telomere length is associated with an increased risk of ovarian cancer in both familial and sporadic cases, particularly for early onset ovarian cancer. In a study performed by Zhou et al., (2012), telomere length variation in normal epithelial cells adjacent to tumor is significantly associated with breast cancer and can be used as a potential biomarker for the local recurrence of breast cancer.

One prominent hypothesis is that telomere dysfunction is one of the key processes underlying the genomic instability observed in primary malignant lesions (Figure 3). A hypothesis concerning telomere dysfunction states that telomeric protection is lost in a small group of normal precursor cells. This loss of telomeric protection results in the fusion of the telomeric regions of different chromosomes, which causes genetic instability through cycles of fusion, bridge formation and chromosomal breakage. Thus, telomeric dysfunction can generate various cytogenetic changes, and the cells acquire a combination of genetic aberrations necessary to initiate carcinogenesis. Genetic instability provides the initial cancer cells with the chromosome changes that disable the suppression of growth and apoptosis, allowing the engagement of metabolic pathways essential for immortal growth (MASER; DEPINHO, 2002). Telomeric dysfunction can be caused by the aberrant length of the telomeric DNA sequence (telomere shortening) and/or the loss of function of a protein associated with telomere (GILLEY et al., 2005).

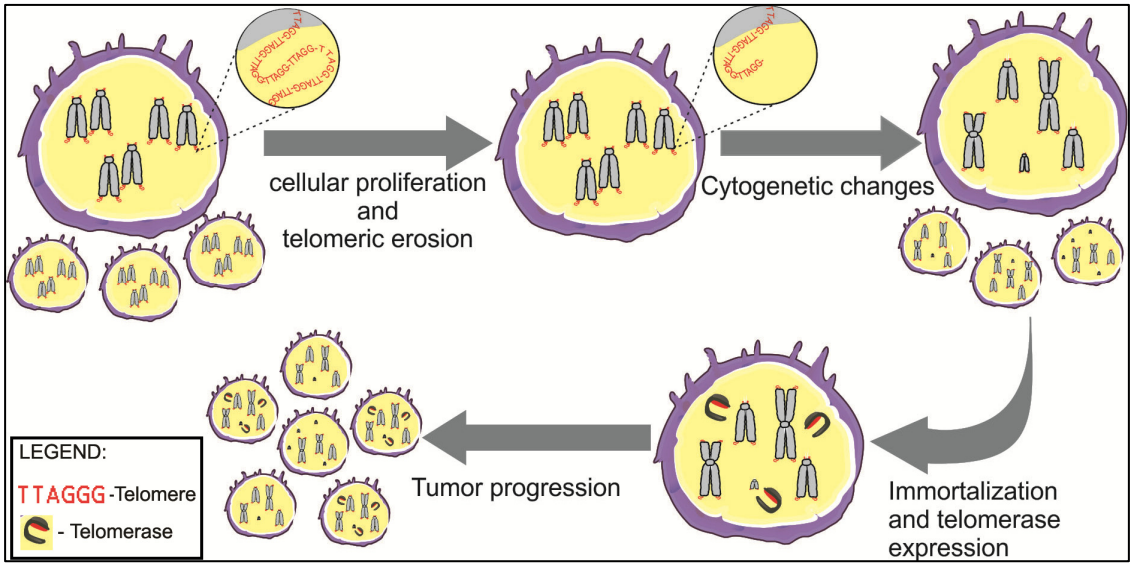

Figure 3. Schematic representation of the role of telomeres in carcinogenesis. A small group of cells loses their telomere protection due to erosion of the telomere. Telomere instability initiates a crisis in which the chromosomes are subjected to cycles of breakage-fusion-bridge so that the cells acquire many chromosomal aberrations and become aneuploid. The cytogenetic changes provide advantageous characteristics such as the deletion of tumor suppressor genes and activation or over-expression of oncogenes. Telomerase over-expression stabilizes the chromosomal tumor markers that originated during the crisis state, enabling cell immortalization and tumor progression (adapted from MASER; DEPINHO, 2002).

When cells are maintained in culture, their telomeres reach a critical length that results in the activation of the Hayflick limit (mortality stage 1 or senescence) and the cells stop dividing. However, the Hayflick limit can be easily broken by inactivation of the growth inhibitory pathways induced by the genes Rb and p53. The continued proliferation of cells after the Hayflick limit has been reached causes exacerbated telomeric erosion and high genomic instability, culminating in a period of massive cell death or cell crisis (stage of mortality 2). Although the crisis is an important barrier to cell immortalization, the massive genetic instability in this stage may be the mechanism by which a few survivor cells acquire the large number of genetic changes required for malignant transformation. These rare cells emerge from crisis through the activation of mechanisms for telomeric maintenance, most commonly by increased expression of telomerase (MASER; DEPINHO, 2002).

Telomerase exists in a ribonucleoprotein complex consisting of a catalytic subunit (TERT) that regulates the activity of telomerase and shows reverse transcriptase (RT) activity, whereas an RNA chain provides the template to add the telomeric sequence (TTAGGG) to the chromosome ends (GILLEY et al., 2005). In humans, telomerase is expressed only in some cells, such as embryogenic cells and active lymphocytes, whereas telomerase activity is detected in 90% of tumors; the other 10% maintain their telomeres through the alternative mechanism of lengthening the telomeres (ALT) (KIM et al., 1994).

Telomerase plays an important role in tumor growth and cell immortalization. Reactivation of this enzyme may be a critical event that promotes tumor proliferation by removing the barrier to telomeric shortening (CHANG et al., 2001). One example comes from a study performed by Takahashi et al. (2003), in which a translocation was found to occur between chromosomes 11 and 22 in Ewing's sarcoma and caused the fusion of the EWS and ETS genes, producing a chimeric protein responsible for telomerase activation in this sarcoma. In their classic study, Chang et al. (2005) also demonstrated the importance of telomerase over-expression when they immortalized an endothelial cell line through transfection and ectopic expression of the telomerase hTERT catalytic subunit. These observations and the frequency and intensity of telomerase expression in human tumors suggest that telomeric maintenance is essential for cell immortalization and that it may be possible to inhibit the growth of cancers by interfering with the action of telomerase (LI et al., 2005). Thus, many strategies have been developed to inhibit telomerase, such as antisense nucleotides, ribozymes and interfering RNAs, for use in therapeutic approaches (GUO et al., 2005).

A hypothetical model to explain the influence of cytogenetic events in carcinogenesis

Considering the data on cell transformation and the hypotheses of other authors, it is possible to propose a model to explain chromosomal changes in tumors that present complex karyotypes, such as the near-tetraploid ones. We hypothesize that initially a normal cell has a faulty mechanisms of replication or checkpoint control, due to physiological or genetic factors or exposure to chemical agents, and becomes polyploid. Extra copies of chromosomes can be generated by endoreduplication, an event frequently observed in tumor cells (BOTTURA; FERRARI, 1963; LIMA et al., 2004). Additional evidence concerning the importance of endoreduplication in carcinogenesis results from the observation that cancer cells exposed to chemotherapeutic drugs that inhibit mitosis utilize endoreduplication to evade apoptosis, and the occurrence of endoreduplication is associated with the development of secondary malignancies (CORTÉS; PASTOR, 2003; CANTERO et al., 2006; PUIG et al., 2008). Davoli and De Lange, (2012) also reported that endoreduplication and mitotic failure occur during telomeric crisis in human cells and promote the transformation of mouse cells.

After increasing the chromosome number of the tumor-initiating cell, the next step toward malignancy would be the acquisition of aneuploidy. According to Mayer and Aguilera (1990), polyploidy *per se* appears to dramatically increase the loss of chromosomes, presumably due to increasing genome instability and the inability of polyploid cells to undergo proper chromosome segregation. Another event that can generate aneuploidy is double-strand breaks (DSBs) in the DNA molecule. When a DSB occurs, the chromosome becomes unstable and needs to be repaired. One way to solve this problem is by joining the damaged chromosomes, but erroneous rejoining of broken DNA may occur, resulting in the deletion or amplification of chromosome material as well as translocations (KHANNA; JACKSON, 2001; JACKSON, 2002). DSBs can result from telomere erosion after several rounds of mitotic cycles or by oxidative stress resulting from inflammation, which causes, among other effects, an

increase in the free radicals at the tumor site (see Figure 4).

Because of double-strand breaks (DSBs), chromosomes destabilize and may suffer from various structural alterations such as fusions, bridges and chromosome breakage. Genetic instability is responsible for the generation of important chromosome malignant markers, which are ubiquitous in the karyotypes of solid tumors. Genetic instability and chromosomal aberrations may provide some advantageous phenotypic changes in the tumor cells, enabling them to evade apoptosis by retaining their proliferation potential. Thus, some rare cells with ideal chromosomal combinations may achieve a malignant phenotype. However, the chromosomes of these tumor cells still undergo unstable replication and segregation, most likely due to telomere dysfunction. This obstacle is overcome by reactivation or over-expression of telomerase.

After chromosomal stabilization by telomerase, cancer cells are able to divide indefinitely, establishing tumors. During tumor progression, many changes occur and only advantageous genotypes are positively selected. Although cellular heterogeneity among the tumor cell population exists, only cells that have the ideal chromosome combination, called tumor stem-cells, are responsible for the perpetuation of cancer.

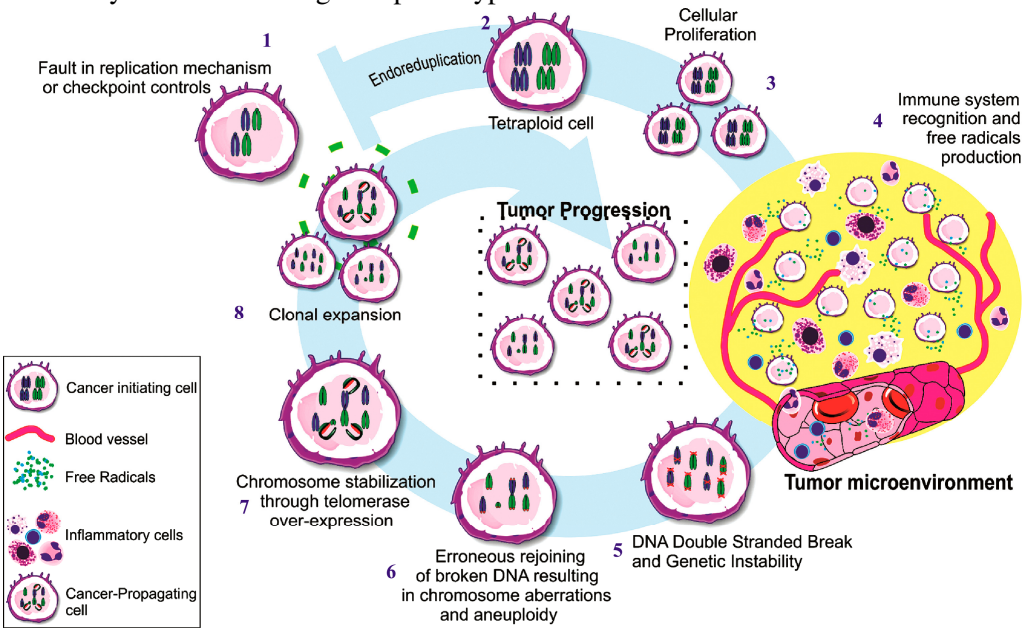

Figure 4. A proposed model for the karyotype evolution of a cancer cell. Due to faults in the replication mechanism or check-point controls, a normal cell becomes polyploid through endoreduplication. After many mitotic cycles, the resulting cells are recognized by the immune system, which tries to eliminate the abnormal cells by producing cytotoxic mediators such as free radicals. These reactive compounds attack DNA, causing double-strand breaks (DSBs) and genetic instability. Attempts to stabilize the DSBs result in the erroneous rejoining of broken DNA, giving rise to aneuploidy. A rare cell with an advantageous near-tetraploid karyotype may possess a better proliferative potential when the chromosomes are stabilized by telomerase over-expression. These specific proliferating cells promote tumor progression through clonal expansion.

CONCLUSION

The data presented in this review elucidate the importance of chromosomal rearrangements in the evolution of tumor as this event is nearly ubiquitous in the malignant cells of most patients. More effort should be given to cytogenetic studies because the evidence indicates that a correct chromosomal balance is crucial to cancer development, and cytogenetic techniques are valuable tools to diagnose cancer and direct cancer treatment. The interface between classic cytogenetics, molecular cytogenetics and molecular genetics must be exploited to generate the data to improve our knowledge of cancer biology.

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RESUMO: As duas visões conflitantes da tumorigênese que são amplamente discutidas são a hipótese da mutação gênica e a hipótese da aneuploidia. Nesta revisão vamos resumir as contribuições da citogenética no estudo das células tumorais e propor um modelo hipotético para explicar a influência dos eventos citogenéticos na carcinogênese, enfatizando o papel da aneuploidia. A teoria da mutação gênica estabelece que mutações específicas ocorrem e mantêm o fenótipo alterado das células de um tumor, enquanto a hipótese da aneuploidia estabelece que a aneuploidia é necessária e suficiente para a iniciação e progressão da transformação maligna. A aneuploidia é considerada um marcador do câncer e esta desempenha um importante papel tanto na tumorigênese, quanto na progressão tumoral. Células aneuplóides podem ser derivadas de células poliplóides, que surgem espontaneamente ou são induzidas por agentes ambientais ou compostos químicos. A instabilidade genética observada em células poliplóides leva a perdas ou rearranjos cromossômicos, resultando em cariótipos variavelmente aberrantes. Devido à grande quantidade de evidências indicando que um balanço cromossômico correto é crucial para o desenvolvimento do câncer, as técnicas citogenéticas são ferramentas importantes tanto para a pesquisa básica, tais como pesquisas para elucidar a carcinogênese, quanto pesquisas aplicadas, como no diagnóstico, prognóstico e escolha do tratamento. A combinação da citogenética clássica, citogenética molecular e genética molecular é essencial e pode gerar uma grande quantidade de dados, aumentando o nosso conhecimento da biologia do câncer, melhorando assim o tratamento desta doença.

PALAVRAS-CHAVE: Tumor. Cromossomos. Instabilidade cromossômica. Técnicas citogenéticas.

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