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Review

Defective mitosis-linked DNA damage response and chromosomal instability in liver cancer

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ABSTRACT

Hepatocellular carcinoma (HCC), the most common form of liver cancer, represents a health problem in hepatic viruses-eradicating era because obesity, type 2 diabetes, and nonalcoholic steatohepatitis (NASH) are considered emerging pathogenic factors. Metabolic disorders underpin mitotic errors that lead to numerical and structural chromosome aberrations in a significant proportion of cell divisions. Here, we review that genomically unstable HCCs show evidence for a paradoxically DNA damage response (DDR) which leads to ongoing chromosome segregation errors. The understanding of DDR induced by defective mitoses is crucial to our ability to develop or improve liver cancer therapeutic strategies.

1. Introduction

Hepatocellular carcinoma (HCC) represents 85% of liver cancer and is associated with chronic liver disease, mainly related to hepatitis B virus (HBV), hepatitis C virus (HCV), and alcohol abuse. However, recent studies identify nonalcoholic steatohepatitis (NASH) as the underlying cause in 20% of patients. NASH is associated with obesity, diabetes mellitus and histologically is characterized by steatosis, necroinflammation, and cirrhosis [1]. However, in recent years several studies have described the onset of HCC in NASH patients who do not have cirrhosis yet. Importantly, HCC diagnosis is underestimated and the disease is often asymptomatic for decades. Based on these epidemiological data and on the role of obesity and diabetes as carcinogenic factors, the efforts in understanding the rising of liver cancer in these patients are fully justified. Hepatocarcinogenesis involves numerous phenotypic and genotypic alterations investing both transformed hepatocytes and stromal components. In particular, the interactions between stromal cells (e.g. myofibroblasts, monocytes/macrophages) and transformed hepatocytes represent a crucial step in the process of tumor progression. These observations have fueled the hypothesis that a boosted hepatocyte turnover occurring in a context of metabolic liver disease contributes to the induction of the chromosomal instability (CIN) that represents one of the key features of HCC tumorigenesis. CIN is sensed as DNA damage and induces a signaling pathway named DNA damage response (DDR), Fig. 1 [2]. The DDR is involved in several aspects of DNA integrity because it affects several cellular processes such as mitosis, senescence, apoptosis.

In this Review we discuss the relationships between abnormal mitosis and CIN with a special focus on how the involvement of DDR can either increase or decrease the sensitivity of the current HCC treatments. This pathway has a central role in mitotic genome segregation, indeed the depletion of DDR components or their abnormal activation during mitosis perturbs the process of accurate chromosome segregation. How various types of mitosis-linked DNA damage engage the DNA damage signaling pathways and repair components is unknown. The effects of activating the DDR during mitosis have remained obscure on the basis that it is difficult to study, however recent findings reveal it can paradoxically lead to deleterious effects on genome stability.

2. From chromosomal aberrations towards cancer

Abnormal chromosome number termed aneuploidy is the distinguishing feature of cancer cells, a state in which cells do not contain an exact multiple of the haploid DNA content, of note the term aneuploidy refers to a static feature. An important aspect in studying cancer aneuploidy is to discriminate between the state of the karyotype and the rate of karyotypic change [3]. Aneuploidy is not synonymous with CIN, some tumors are stably aneuploid with a highly abnormal but fairly

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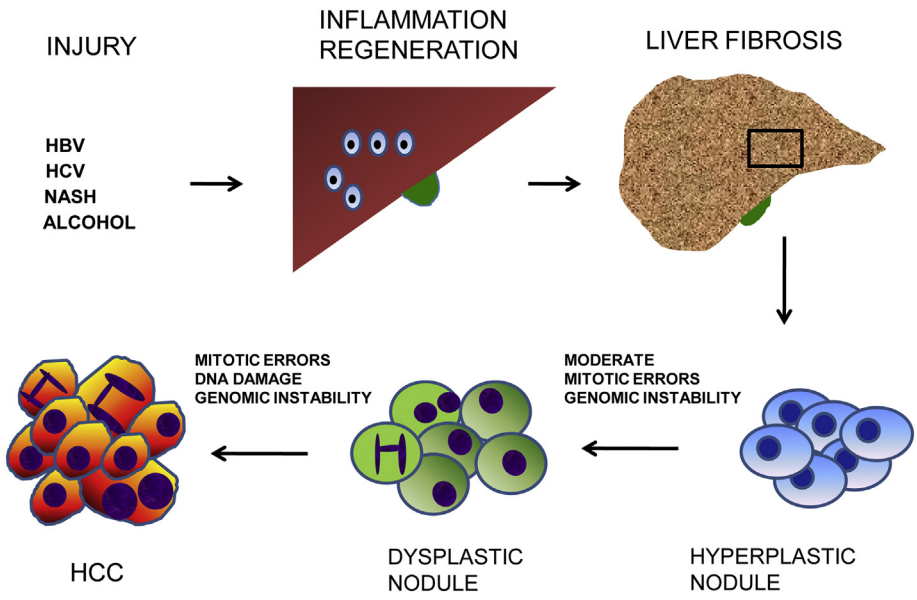

Fig. 1. The neoplastic evolution of HCC proceeds through a multi-step histological and genetic process. Hyperplastic nodules of regenerating hepatocytes have normal karyotype and represent a potential first step towards HCC. These lesions can progress to dysplastic nodules which have activated DNA damage signaling pathway with abnormal chromosome features including numerical and structural alterations. These dysplastic nodules can evolve to stable and unstable HCC marked with recurrent regions of copy number change and allelic imbalances.

uniform karyotype. In other tumors, an increased rate of CIN generates diverse karyotypes within a tumor, the term chromosomal instability describes a rate of change i.e. a dynamic feature of chromosome pathophysiology [4]. This affects chromosome number and structure and is a characteristic of many cancer types including HCC, it is also associated with the formation of extranuclear bodies that contain damaged chromosome fragments or whole chromosomes [4,5]. Such micronuclei were identified in regenerative and dysplastic nodules of the liver indicating that CIN can be acquired already in early stage of hepatocarcinogenesis. Polyploidy, an increase in DNA content by whole-number multiples of the entire set of chromosomes, is also thought to be a possible mechanism that contributes to liver carcinogenesis [6]. Polyploid cells are formed during oxidative damage to the liver and regrowth of the liver after partial hepatectomy and are associated with a pronounced increase in the population of polyploid cells development in otherwise diploid organisms. A growing amount of evidence indicates that polyploid cells also arise during a variety of pathological conditions. Genetic instability in these cells might provide a route to aneuploidy and CIN and thereby contribute to the development of cancer [7,8].

Overall, chromosome alterations are classified in two groups: numerical aberrations and structural aberrations. In numerical type, usually, a whole chromosome is gained or loss due to cell division errors. This results in deviation of chromosome content of the cell from normal diploid state. Structural abnormalities include several chromosomal rearrangement such as translocations, inversions, deletions and gene copy-number amplifications and mostly produced by un- or miss-repaired breakages of DNA [9]. In 1890, the German pathologist David von Hansemann identified that “the cancerous process begins with an altered division of chromosomes after metaphase in a local tissue cell” and proposed it as one of the main factor in tumorigenesis. In general the karyotypes of cancerous cells have been usually considered with 50–90 chromosomes and in a complex form harboring a variety of structural chromosomal changes. Such chromosomes content is very important in cancer cell homeostasis. Of note, such karyotypic heterogeneity varies among the different cells of a given cancer and also from one cancer to another and may also differ during stages of cancer initiation, progression and invasion [10]. In some cancers karyotypic pattern is specific providing the basis for chromosomes as diagnostic tool in cancer. The best example is translocation of chromosomes 9:22 (Philadelphia chromosome) in 90% CML patients. By means of fluorescent in situ hybridization, in 1990, Christoph Lengauer showed that aneuploidy is a progressive phenomenon which is due to CIN so it is not just a passive and marginal product of transformation [9]. However, much literature uses the aneuploidy and CIN terms in a common meaning and produces ambiguity. It has been evidenced that chromosomal aberrations can accelerate the clonal evolution of cancer cells under selective pressure by gain or loss of genetic materials. Besides, it can also play an important role producing a balance and homeostasis between cellular life and adaptation [11]. Liver cancer is not exception from these events and HCC cells are also cells with aberrant chromosomes which harbor variety of genetic rearrangements including translocations, deletions and gene amplifications. Some of these modifications are unique to special sub-types of HCC while the others are common in types, these findings suggest that the observed down-regulation of SLU7 in the cirrhotic liver could participate in the induction of DNA damage, aneuploidy and genome instability which in chronic liver injury precede HCC development [12]. Recently, a study has linked caspase-8-dependent apoptosis to HCC development via proliferation-associated DNA damage. Proliferation-associated replication stress, DNA damage, and genetic instability are detectable in chronic liver disease (CLD) before any neoplastic changes occur both in human CLD and murine CLD models, the study has identified that increased hepatocyte apoptosis resulting in regenerative proliferation, high DNA replication rate and DNA damage are important determinant of hepatocarcinogenesis [13].

Along these lines, etiological factors of CLD and HCC represent important features of CIN drive as well. Meaningfully, CIN has been associated with TP53 mutations and related to HBV infection, HCCs with a high histological grade show a high TP53 mutation rate, whereas HCC with a low histological grade show a low TP53 mutation rate. Genetic alterations in five Wnt pathway genes (CTNNB1, AXIN1, FGF19, RSPO2, and APC) are significantly associated with the absence of HBV infection. This last group of tumors includes HCV infection and NASH-related HCCs.

3. Defective spindle-kinetochore-chromosome structures

Due to the importance of spindle-kinetochore interaction during cell division, it is not surprising if any defects in spindle attachment is connected to CIN. Such errors are common in cell divisions as the kinetochores connect to the microtubules by chance [14]. During the cell division, to ensure the proper chromosome segregation over the two daughter cells, each kinetochore has to connect to a single spindle pole through a single kinetochore fiber, the process is termed as amphitelic attachment and regulated under the activity of spindle assembly

checkpoint (SAC). In fact, kinetochore is a complex disc-shaped protein that connects the centromere into the spindle microtubules through the cell division [15]. Besides, other actions have been attributed to the kinetochores. These consist of establishment of centromeric heterochromatin mediating the sister chromatids adhesion through the cohesin and sister chromatids segregation during anaphase. Of note, in the absence of proper chromatids-kinetochore-microtubules attachment, SAC error correction machinery induces the mitotic delay to prevent starting the anaphase [16]. This happens through inhibition of anaphase promoting complex (APC) to attach to its activator CDC20. In a recent study by Tauchman et al., they showed that hyper-stable kinetochore–microtubule attachment satisfies the 'wait-anaphase' signal produced by the SAC [17]. Several defects have been reported in HCC cells in which kinetochore structure or orientation are damaged, these include monotelic, syntelic and merotelic attachments [18,19]. In monotelic attachment error, one of the sister kinetochores attaches to microtubules from one pole, whereas the other remains unattached to any microtubules. In such condition, the unattached kinetochores are recognized by mitotic checkpoints producing anaphase initiation delay through the inhibition of anaphase promoting complex. In syntelic attachment, both sister kinetochores interact to the microtubules originating from the same pole. Merotelic kinetochore orientation is an error in which a single kinetochore is bound by microtubules emanating from both spindle poles. In human, several proteins like pRb, the kinesin-related MCAK and Aurora B kinase have been recognized to correct or prevent the merotelic attachment errors but is not clear if their absence results in kinetochore architecture changes towards merotelic orientation or CIN [20]. Growing body of evidences demonstrate that spindle defects especially merotelic structures can increase the chromosome instability and boost the harboring cells of such error towards tumorigenesis. In HCC cells variety of spindle defects has been reported, Saeki et al. have demonstrated that impaired mitotic assembly checkpoint in HCC is associated with aneuploidy and aneuploid HCC cells (62.5%) harbor the loss of this checkpoint [21].

4. Aberrant spindle morphology

Mitotic spindle is an apparatus which forms during cell division and promotes sister chromatids segregation through the anaphase. The mitotic spindle is composed of a variety of proteins among which tubulins are predominant. The chromosomes attached to the tubulins via the kinetochore proteins actively monitor spindle formation and prevent premature anaphase onset. It has been evidenced that aberrant morphology or orientation of mitotic spindle can be connected to chromosome aneuploidy. Multipolar mitotic spindles are common characteristics of many solid tumors which eventually lead to chromosome segregation errors and CIN. Centrosome abnormalities play an important role in formation of such multipolar mitotic spindles and centrosome amplification is one the main cause of multipolar spindle and can occur in TP53 mutated-HCC or HBV-infected hepatocytes [22]. Altered expression of a variety of genes have been reported to mediate centrosome amplification. As an example, Nelsen et al. showed that transient overexpression of cyclin D1 in normal hepatocytes results in supernumerary centrosomes and abnormalities of the mitotic spindle [23]. Furthermore, it has also been evidenced that nucleophosmin/Ran/Crm1 complex mutations or its disruption due to HBV infection, play an important role in centrosome duplication [22].

5. The mitotic checkpoint

To ensure the exact segregation of sister chromatids during mitosis, eukaryotic cells express a variety of regulatory proteins termed mitotic checkpoint which act in specific points along the cell cycle phases and guarantee the proper division of the cells, Fig. 2 [27–26]. Therefore, it is not surprising that any defect in mitotic checkpoint is connected with aneuploidy, CIN and cancer [26]. Decreased expression as well as

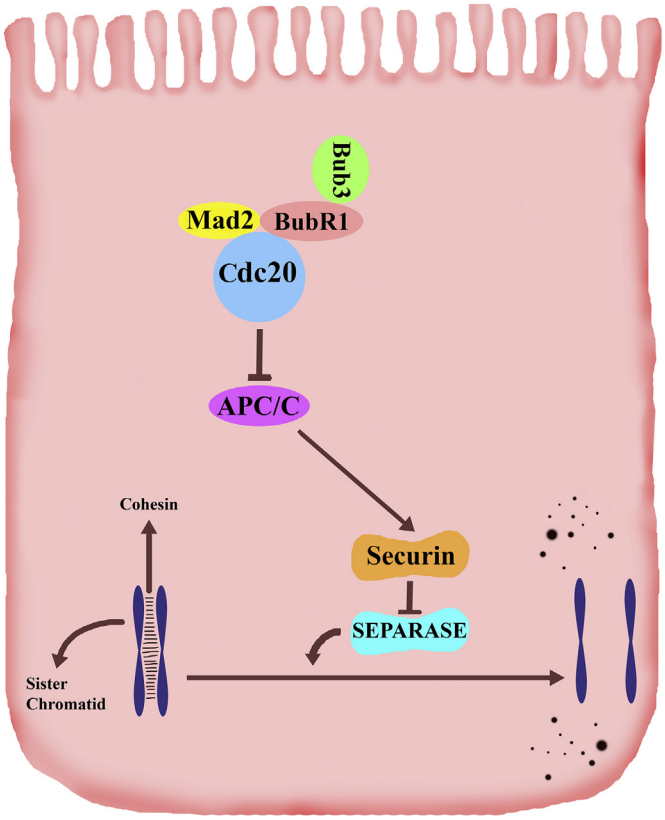

Fig. 2. The mitotic checkpoint signaling. The signal generators of this checkpoint are unattached kinetochores. Kinetochore assembly recruits MAD2 (mitotic arrest deficient homologue 2), BUB1 (budding uninhibited by benzimidazole), BUB3, BUBR1 to unattached kinetochores. The kinase activity of BUBR1 is essential for checkpoint signaling. The actions of all of these components are required for tight association with CDC20 (cell division-cycle 20) in unattached kinetochores, preventing it from activating the anaphase-promoting complex/cyclosome (APC/C) and thereby inhibiting ubiquitylation of securin. Separase, the protease that cleaves the cohesins that hold sister chromatids together, is inhibited by binding to securin.

mutations in mitotic checkpoint genes have been observed in HCC and other tumors, however these events are rare. Mitotic checkpoint overexpression is a more frequent observation in human tumors and is sufficient to generate CIN in vivo and in vitro. Mitotic checkpoint overactivation results in a prolonged mitosis and an increased incidence of merotelic attachments and lagging chromosomes. Dysregulation of mitotic checkpoint has been repeatedly reported in HCC cells which eventually lead to aneuploidy and rearrangements in these cells. The master regulators which have been described in HCC are BUBR1, MAD1/2, BUB3, and CENP-A/E [27].

Human BubR1 is a serine/threonine protein kinase which is involved in kinetochore activity to determine if chromosomes have achieved alignment at the spindle equator [28]. Overexpression of BubR1 at both mRNA and protein levels, has been described in HCC especially in HBV antigen positive cases and was associated with larger tumor size, higher histological grade, advanced pathological stage, poor recurrence-free survival and worse prognosis [24]. BUB3 is one the key factor in SAC and delays the start of anaphase restraining kinetochore localization during metaphase [29]. The impairment of Bub3 and its family members Bub1 has been reported in HCC. Mitotic arrest deficient 2 (MAD2) is one the essential mitotic checkpoint which is involved in metaphase-anaphase transition. In particular MAD2 gene is a component of spindle assembly checkpoint that prevent anaphase until all sister chromatid pairs have become bipolarly attached. The findings using experimental mice models are conflicting, Mad2 overexpression

induces aneuploidy and tumorigenesis in mice model [30]. Conversely, down regulation of Mad2 has been reported in human HCC samples and is associated with defective mitotic responses [31]. However, knocking out of both Mad2 and p53 in experimental mice model have been connected with phenotypes resembling HCC including hepatocyte regeneration, genomic instability and fibrotic tumor microenvironment. Recently, Foijer et al. have described the consequences of deleting MAD2L1 in murine thymocytes and hepatocytes. In both cell types, rapidly growing tumors arise from cells that have lost Mad2l1 expression, tumorigenesis is promoted in both thymocytes and the liver by heterozygous or homozygous deletion of Trp53, establishing the pivotal role of Trp53 as a potent tumor suppressor [32].

Centromere protein E (CENP-E) and centromere protein H (CENP-H) are both critical for mitotic checkpoint and move the replicated chromosomes towards metaphase plate during the mitosis [33–35]. Reduced expression of CENP-E has been reported in HCC cell lines and reduced expression increases the rate of aneuploidy in the cells [34].

Centromere protein A (CENP-A) is one of the important protein in centromere formation and epigenetic process. CENP-A is a Histone H3-like protein, which is required for the proper formation of centromeres and kinetochores. CENP-A has been reported to be overexpressed in HCC samples and mRNA targeting of this gene inhibits the tumor growth in HCC cells [36]. CENP-A overexpression leads to ectopic kinetochore formation, resulting an increased numbers of erroneous microtubule-centromere attachments upon mitotic entry and chromosome segregation defects. Liu et al. showed that overexpression of CENP-A has been associated with Hepatitis B virus X protein in HCC tissues [25].

Cell division cycle protein 20 (CDC20) is one the key element in activation of anaphase-promoting complex/cyclosome (APC/C) to initiate sister chromatid separation [37]. Overexpression of CDC20 has been evidenced to be connected with tumor differentiation and progression of HCC [38].

Anaphase promoting complex/cyclosome (APC/C) is one the main element in regulation of anaphase during the mitosis. The APC/C's main function is to trigger anaphase beginning by tagging specific proteins for degradation. The three major targets for degradation by the APC/C are securin and cyclins. Securin releases separase (a protease) after being degraded. The separase triggers the cleavage of cohesin, the protein complex that binds sister chromatids together. It has been documented that altered functions of APC/C play an important role in genomic instability and tumorigenesis.

6. DNA damage checkpoint and DNA repair pathways

The defective maintenance of organized and proficient DNA is detrimental for cellular equilibrium. Induction of DNA double-strand breaks (DSBs) in normal cells by ionizing radiations or radiomimetic drugs activates a DNA damage response that produces cell cycle arrest, DNA injury repair, and progression of cell cycle. Several proteins are connected with DNA damage signaling and repair, localizing at broken sites in focal structures termed foci [39]. DSBs are detected by the multiprotein complexes Mre11–Rad50–Nbs1 (MRN) and Ku70–Ku80, that successively recruit the PI3-kinase–like kinases (PIKKs), such as ataxia telangiectasia mutated (ATM), and DNA-dependent protein kinases (DNA-PKcs). A main PIKK target is the C terminus of the histone variant H2AX, whose phosphorylated serine 139 (S139) is stated as γH2AX [40]. Phospho-S139 of H2AX is then recognized by BRCA1 C-terminal domain (BRCT) domains of the DDR protein MDC1 (mediator of DNA damage checkpoint 1). ATM-mediated phosphorylations to DSB sites contributes to form γH2AX-MDC1 foci. MDC1 phosphorylated by ATM recruits the RING-finger ubiquitin E3-ligase RNF8, which, at the side of another ubiquitin E3-ligase, RNF168, produces DSB-associated ubiquitylations on histones H2A and H2AX that, in turn, promote accumulation of p53-binding protein one (53BP1) and breast cancer type 1 susceptibility protein (BRCA1). DDR proteins gather in a spatiotemporal manner at sites of DNA breaks instead of being recruited as a preassembled macromolecules [41]. Furthermore, modifications such as phosphorylation regulate the structure and activity of DDR target proteins providing docking sites for other DDR proteins. In most respects DDR proteins display phospho-binding motifs such as BRCT (breast cancer C-terminal) or FHA (forkhead-associated domain) that contribute to mediate the phospho-dependent assembly of DDR protein complexes, Fig. 3. The DNA damage checkpoint kinase CHK2 is a central effector of this response to DSBs. Human Chk2 is a 543-amino-acid protein that consists of a N-terminal SQ/TQ cluster domain (SCD), a central forkhead-associated (FHA) domain and a C-terminal serine/threonine kinase domain. The SCD consists of multiple SQ/ TQ (Ser-Gln/Thr-Gln) motifs with Thr68 being the primary site to be phosphorylated in response to DNA damage followed by phosphorylation of Thr387 in the activation loop of the kinase domain [42]. Surprisingly, CHK2-null mouse mutants are viable and do not develop spontaneous tumors, even more wild type and $ATM^{+/-}$ mice develop HCC, at 9–12 months, whereas $ATM^{-/-}$ mice remain refractory to diethylnitrosamine induced HCC up to 15 months [43]. These observations deserve further investigation regarding the precise mechanisms and functions of DDR proteins in mitotic cells. The overexpression and activation of the DDR proteins during mitosis perturbs several aspects of mitosis, including mitotic timing, anaphase and cytokinesis inducing defective chromosome segregations [44]. Kim et al. reported that γH2AX foci were significantly increased in HBV-related liver cirrhosis and HBV-related HCC. Importantly, dysplastic nodule showed a significantly higher level of γH2AX compared with HCC [45]. HCC mechanism of progression is less well defined than that of other cancer types. Hyperplastic nodules have normal karyotype and represent a potential first step towards HCC. These lesions can progress to dysplastic nodules, which have abnormal chromosome features including numerical and structural alterations. Loss of certain tumor suppressors

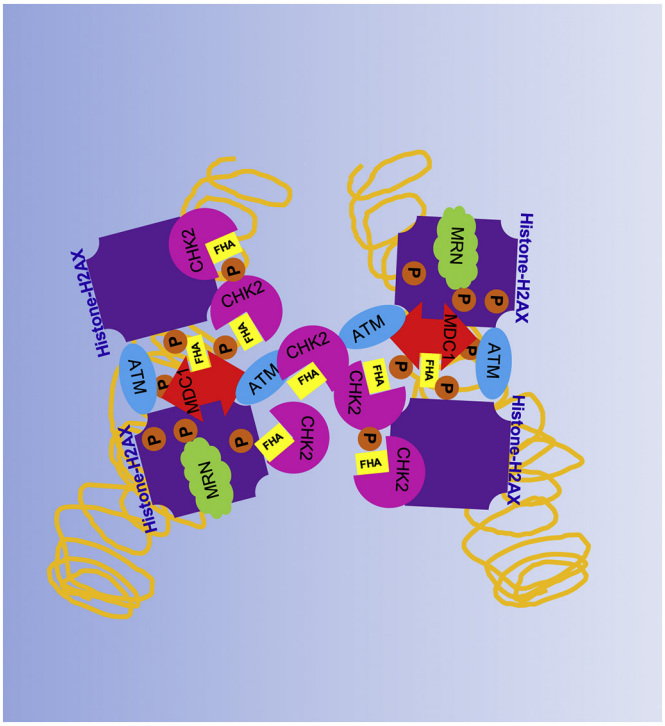

Fig. 3. Spatial organization of DDR protein accumulation at DNA DSBs. DDR proteins accumulate at DNA double strand breaks (DSB) sites involving MDC1, which binds histone H2AX, the MRN complex, and ATM kinase, which phosphorylates additional histone H2AX molecules. ATM-dependent phosphorylation of MDC1 on Thr-Gln-X–Phe (TQXF) motifs creates binding sites for the FHA domain of CHK2.

or gain of specific oncogenes promote cell division and this coincides with stochastic defective mitoses which can cause DNA damage [46,47]. The scenario is conceivable considering that massive deregulation of mitosis is incompatible with cell survival and can even be tumor suppressive [46]. Therefore, subtle rather than massive mitotic defects and DNA damage are expected to underlie CIN in HCC, allowing a tolerable level of chromosome defects. Hepatocarcinogenesis with lagging chromosomes elicits the expression of DNA damage response protein Chk2, the overexpression of Chk2 and its mislocalization within structures of the mitotic spindle contribute to sustain cell division and chromosomes missegregation. DDR proteins must localize to the right place at the right time to ensure the efficient signaling and repair of DNA damage. Thus, in chromosomally unstable cancers activation of the DDR may be a insidious phenomenon due to the intrinsic and enduring level of DNA damage during mitosis [2]. More importantly, these findings raise the possibility that mitoses in cancer cells have lost the fine tuning of DDR proteins promoting genomic instability and linking mitosis, DNA damage to numerical and structural chromosomal aberrations.

7. The influence of chromosomal instability on HCC therapy

Low sensitivity of HCC cells to chemotherapy is partly due to multidrug resistant phenotype (MDR) of these cells. Although the exact mechanism of such resistance is not clear, some signaling pathways including drug efflux pump, HIF1-α signaling and CIN play an important role in modulation of HCC-associated MDR [48]. It has been evidenced that chromosomal aberrations confer the MDR-phenotype in cancer cells and eventually lead to treatment failure. Regarding HCC treatment a variety of signaling pathways have been exploited, however conventional oncogene-directed drugs are not usually effective. It should not to be ignored that chromosomal alterations yield cancer cells resistant against these agents, indeed the effectiveness of the drugs is correlated with the degree of CIN. Therefore, targeting CIN-linked signaling pathways seems to be an interesting approach. Immune-checkpoint blockade represents a therapeutical tool with tremendous potential in treatment of several form of cancer including HCC, therefore whether and how CIN influences immune evasion is of pivotal interest to improve the efficacy of immune-checkpoint blockade. Indeed, the effectiveness of immune-checkpoint inhibition is associated with mutations which cause single amino acid substitutions as reported in patients with melanoma, NSCLC, or cancers with DNA-mismatch-repair deficiencies. Along these lines, CIN-producing pathways are welcomed as most of the cancer cells are chromosomally altered. Nevertheless, CIN can be also an obstacle producing defects in neoantigens editing, defects in antigen presentation and inhibition of tumor infiltration, or cytotoxic activities of immune cells [3]. Additionally, DDR and CIN cause derangement of metabolic attitude of cancer cells boosting glycolysis in the presence of dysfunctional mitochondria. Therefore, cancer cells might compete with T cells for glucose in tumors, and restricting T cell glucose metabolism causes lymphocyte defects. Thus, therapies targeting DNA damage response/CIN that decrease glucose use by cancer cells could make glucose available for T cells and enhance immune-effector functions to limit tumor growth [49]. Revealing the cytogenetic signature of cancer cells is fundamental in understanding the histogenesis, morphology and biology of cancer cells, therefore cancer research have to turn its attention to uncover the specific cytogenetic patterns of different cancer cells to evaluate them as possible prognostic, diagnostic and therapeutic indicators. In fact, CIN can produce heterogeneous gene expression in the cancerous cells and affects the treatment responses by altering gene regulatory interaction and varying protein concentration. The cytogenetic characterization of solid tumors is challenging due to techniques difficulties, although recent advances in functional genomics such as NGS technologies leverage these difficulties. Nowadays, genomic loss and gain aberrations are easily detectable in clinical samples. The issue seems promising in diagnosis of a rare and lethal subtype of HCC called fibrolamellar HCC (FL-HCC), tumor cells posses a 400 kb deletion on chromosome 19 which produces in frame fusion of DNAJB1 gene encoding heat shock protein B1 and cAMP-activated tyrosine kinase PRKACA as a specific cancer driver [50]. Furthermore, other putative druggable genes were reported in HCC through the integrated genomic approaches, these include genes Wnt/β-catenin, JAK-STAT, oxidative stress and chromatin remodeling signaling pathways.

8. Conclusions

The relevance of the DDR during defective mitosis and CIN is only beginning to be revealed including the proteins and the mechanisms involved in the complicated process of chromosome segregation. In normal cells with defective chromosome segregations and DNA damage, the DDR promotes clearing of cells through a p53-dependent mechanism. In cancer cells, which often lack a functional p53-signaling pathway and a proficient apoptosis, overexpression and activation of the DDR proteins and their mislocalization within the mitotic spindle transform DNA damage into structural chromosomal defects. A deep comprehension of this process is essential to our understanding of how DNA damaging therapies influence genomic integrity and how to modulate the mitotic DDR apparatus to improve classic chemotherapy and the promising immunotherapy.

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Conflict of interests

The authors have declared that no conflict of interest exists.

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