Review Article
Unbalanced replication as a major source of genetic instability in cancer cells

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Abstract: The origin of genetic instability in tumors is a matter of debate: while the prevailing model postulates a mutator phenotype resulting from an alteration in a caretaker gene as a prerequisite for genetic alterations leading to tumor formation, there is evidence against this model in the majority of cancers. A model for chromosomal instability should take into account the role of oncogenes in directly stimulating DNA and cellular component replication, creating aberrant structures when overexpressed. I will distinguish here two distinct mechanisms for the genetic instability of tumors: primary and secondary. Primary genetic instability is dependent on the inactivation of genes involved in maintaining genetic stability (caretaker genes), whereas secondary genetic instability is dependent on genes involved in tumor progression, i.e. oncogenes and tumor suppressor genes of the gatekeeper type. Secondary genetic instability, the most frequent condition, can be explained by the fact that some of the genes involved in tumor progression control replication of cell structures from within, leading to replication unbalance.

Keywords: Genetic instability, tumorigenesis, oncogenes, tumor suppressor genes, DNA replication, cell replication, replication unbalance, chromosomal instability

Introduction

Acquisition of the multiple characteristics of cancer cells depends to a large extent on the accumulation of alterations in their genomes [1]. Although this succession of alterations might be partly explained through serial clonal expansion without inherent defect in the cellular replication machinery [2], there is ample evidence that cancer cells display intrinsic genomic instability manifested by high rates of gene amplification [3, 4]. Most cancers are also characterized by chromosomal instability [5] (CIN) leading to changes in chromosome numbers, (whole chromosome aneuploidy [6]); while a minority of tumors display microsatellite instability (MSI) [7, 8] or, exceptionally, increased frequency of point mutations [9]. The term CIN is now frequently used to denote the propensity to acquire not only aneuploidy but also gross genomic alterations such as gene amplification or deletion, and chromosomal translocation [6, 10]. Throughout this article, the term structural CIN (s-CIN) will be used to denote this latter con-

dition. The mutator hypothesis [11] states that a mutation in a caretaker gene [12], the function of which is to maintain genetic integrity, is required for every cancer to develop, because the number of mutations required for cancer development is generally too high to be likely achieved through the «normal» mutation rate. Indeed, the mutation rate of normal cells is considered as very low [13], except in the case of the lymphoid lineage. Tumors originating from this lineage use the machinery required for antigen receptor diversification for generating the steps involved in cancer progression [14], a specific situation which will not be discussed in this review. Apart from these lymphoid tumors, the prevailing model postulates that inactivation of two caretaker alleles is the mechanism leading to genetic instability in cancer cells [12]. From currently available information concerning the genetics of tumors however, it appears now that the mutator hypothesis is certainly valid for a limited number of cancers, whereas this model does not account for most of them, where genomic instability can then be consid-

ered as "secondary" with respect to oncogenic transformation. This means that some of the genes that are responsible for tumor progression are also involved in genetic instability, alone or in association. In this review, I will argue that considering cancer cell proliferation as the consequence of uncontrolled replication illuminates the genesis of CIN.

Primary genetic instability

Mutations in DNA repair genes seem to fulfill the postulate required for the mutator hypothesis [11]. The first well-documented example of this mechanism is the MSI induced by mutations in DNA mismatch repair (MMR) genes. MSI can be found in both hereditary and sporadic cancer. Lynch syndrome [15], or hereditary non-polyposis colon cancer (HNPCC), is linked to a germline mutation in one of the MMR genes [16-19]. As MMR genes are indisputably caretaker genes, and as the germline origin of the MMR mutation identifies it as the first event, there seem to be little doubt that cancer development follows the mutator scheme. In addition, there is evidence that inactivation of the wild-type allele is required for tumor formation, supporting a two-hit mutator model [20]. However, it has been found that somatic MMR loss is often preceded by a number of somatic oncogenic mutations, suggesting a more complex evolution [21]. In addition, patients with a dominant negative mutation [22] leading to MMR deficiency in their normal cells do not develop more often colorectal cancer than heterozygous patients with gene deficiency [23]. A somewhat similar observation could be made in mice deficient for MMR genes, where it appeared that tumorigenesis is not an obligate consequence of DNA replication errors and, in the case of some MMR genes, might be related to other functions [24].

In sporadic colorectal cancers, an epigenetic, rather than a genetic mechanism, is involved in the inactivation of the two alleles of one particular MMR gene, MLH1, which may be a consequence of promoter hypermethylation [25]. MMR may also be related to overexpression of the MSH3 gene, as a consequence of gene amplification [26]. Since both processes, hypermethylation and gene amplification, are frequently observed during cancer progression, it has to be taken into account that these processes do not necessarily follow from the mutator model, but might be more likely a conse-

quence of oncogenesis.

Inactivation of MMR genes is indicative of the existence of a pathway consistent with the mutator hypothesis, but this pathway is uncommon in sporadic cancers. Other familial cancers, some of them with CIN, such as those related to BRCA1 or BRCA2 [27] involve a caretaker defect. However, it is now clear that most sporadic cancers do not involve primary genetic instability. Two arguments support this conclusion. First, mutations in caretaker genes are infrequent in non-familial cancers, which make up the large majority of cancers [28], although one may argue that all caretaker genes are not yet identified. More importantly, in mouse models, inactivation of caretaker genes [24, 29] is less efficient than introduction of oncogenes [30-33] or inactivation of anti-oncogenes [34-36] in the germ-line for the induction of tumors, in contradiction with the mutator hypothesis [11].

Secondary genetic instability with chromosomal structural alteration: role of oncogenes and tumor suppressor genes

Here, I will consider the genetic instability occurring during tumor progression. Results showing that a limited set of oncogenes is sufficient to confer tumorigenicity after introduction in a cell culture [37], and that, as mentioned above, they more frequently cause tumors than does inactivation of caretaker genes in the mouse germ-line, can be taken as evidence to indicate that the caretaker pathway is not necessary for tumorigenesis. In addition, although it is possible to produce tumor cells which do not show widespread genomic instability [38], most of the tumors originating through this oncogenetic pathway display CIN, unlike cancers related to the MMR pathway.

It has been claimed that oncogenes cannot be responsible for initiating the CIN phenotype based on the argument that MMR negative tumors, which are karyotypically stable, have mutations in the same oncogenes and tumor-suppressor genes as CIN tumors and have similar stage-specific growth and progression characteristics [39]. However, both assertions are wrong, as discussed in detail by Perucho [40].

Genes whose inactivation favors cancer formation are called tumor suppressor genes. Two classes of tumor suppressor genes are usually

distinguished: caretaker genes and gatekeeper genes [12]. Caretaker genes, as mentioned above, maintain genome integrity, whereas gatekeeper genes prevent uncontrolled growth. The tumor suppressor gene p53 possesses both functions, as it induces apoptosis or growth arrest in response to DNA damage [41]. One argument that it works mainly as a gatekeeper gene [42] rather than as a caretaker gene [43] is that p53 mutation is often a late event in tumor development [44, 45]. As proposed by Halazonetis et al. [46], p53 acts as a barrier against tumorigenesis in precancerous lesions. P53 protein plays a role in the control of cell growth, by inducing growth arrest through stimulation of an inhibitory pathway and inhibition of oncogene expression, or by inducing cell death [42, 47]. More specifically, p53 represses c-myc expression by various mechanisms [48-50], whereas mutant oncogenic p53 activates c-myc expression [51]. As overexpression of c-myc is an inducer of p53 [52, 53], one of the functions of the wild-type p53 tumor suppressor gene might be to counteract the effect of c-myc.

Oncogene induced overreplication as a cause of chromosomal alteration

In the model proposed by Halazonetis et al. [46], genetic instability is induced by DNA double-strand breaks (DSB) generated as a consequence of oncogene induced replication stress. Although it has been clearly shown that oncogene activation induces DNA DSB [54, 55], it is not yet clear whether this is the only way for oncogenes to induce genetic instability, and there is still a need to find a link between oncogene activity and DNA DSB. In 1986, Schimke and colleagues proposed a model accounting for most of the chromosomal rearrangements and aberrations found in cancer cells [56]. In this model, which was based on Varshavsky's idea of "replicon misfiring" [57], different chromosomal alterations, including gene amplification, could be explained by recombination of overreplicated DNA strands. By the end of the eighties, the c-myc gene was a good candidate to mediate overreplication, because it was thought to be involved in DNA replication [58-61] and it was known to be overexpressed in cancers [62]. One of the predicted [63] effects of the myc gene, stimulation of gene amplification, could be tested using a Methotrexate selection [64] assay combined with an inducible myc construct. In agreement with the prediction,

it was shown that myc overexpression was able to stimulate dihydrofolate-reductase gene amplification [63]. However, the model of myc induced overreplication met little success for the following reasons: first, myc was no longer considered as involved in DNA replication [65], but only as a transcription factor with multiple targets [66]; secondly, the abandonment of the overreplication model as a consequence of lack of direct support [67] and its replacement by the breakage-fusion-bridge model (BFB) model for gene amplification [68] left the effect of myc on gene instability, although largely confirmed in different assays [69-72], unexplained. An alternative model proposed that myc effect would involve reactive oxygen species (ROS) [73]. However, incubation with antioxidant protected against myc induced genetic instability only in serum deprived cells (reviewed in [74]) and myc can induce DNA breaks independently of increased production of ROS [75]. Oxidative stress may also lead to a myc-mediated response [76], questioning the causal relationship between myc, ROS and genetic instability. Another problem with this model is that one would expect cancer cells to be hypermutable in terms of point mutations if ROS are involved, since endogenous oxidative stress induces primarily base damage [77]. Actually, while secondary genetic instability is associated to a high rate of gene amplification [3, 4], it does not involve increased point mutation rate [78, 79].

In a turn of events, recent findings have lent support to the overreplication model as a consequence of myc overexpression. First, a recent proof of a direct involvement of myc in DNA-replication has gained a large acceptance [80]. Interestingly, this role might be related to its association with the minichromosome maintenance (MCM) proteins, which are involved in replication licensing [81]. In parallel, it has been found that myc, as a transcription factor, stimulates, rather than inhibits, the expression of DSB repair genes [82]. Then, the development of a system to detect early amplification events arising from re-replication has indicated that, indeed, re-replication could lead to gene amplification [83]. In addition, duplications and higher order amplifications in direct repeat, which are incompatible with the BFB, model may be prevalent in cancers [84]. Therefore, although other mechanisms have been considered for the induction of myc induced genetic instability [74, 85], the overreplication model seems to be the

Table 1. Involvement of oncogenes and gatekeeper tumor suppressor genes in genetic instability.

Oncogene or Tumor suppressor gene	Involvement in tumors	Type of genetic instability associated with dysfunction	Putative mechanism for genetic instability
myc	Overexpression (amplification, translocation, or most often, indirect)	s-CIN	Overreplication (S phase stimulation)
ras	Mutation altering the aminoacid sequence Overexpression	s-CIN w-CIN?	Overreplication Control myc activity? Centrosome amplification through cyclin D1
Cyclin D1	Overexpression (amplification, translocation, or most often, indirect)	s-CIN w-CIN?	Overreplication (S phase stimulation) Centrosome amplification
Cyclin E	Overexpression (amplification, or most often, indirect)	s-CIN	Impairment of S phase progression Forcing premature S phase entry under conditions of nucleotide deficiency
p53 mutant	Mutation altering the aminoacid sequence + overexpression		
and		s-CIN, w-CIN	Overreplication (control of myc ?) Loss of mitotic checkpoint Centrosome amplification
p53 wild-type	Lack of expression		Tolerance to aneuploidy
APC	Lack of expression (most often)	s-CIN? w-CIN	Control of myc through b-catenin Role in chromosome segregation
Rb	Lack of expression (gene inactivation, or most often, indirect)	w-CIN s-CIN?	Forcing premature S phase entry under conditions of nucleotide deficiency Centrosome amplification

? is used when the corresponding type of genetic instability, although predictable, has not been clearly demonstrated

most appropriate. Evidence for re-replication upon overexpression of c-myc has been found in some, but not all, instances [53, 80].

As Schimke's model predicts that overreplication would lead to most of the abnormalities associated with s-CIN, myc overexpression may be a driving force in this type of genetic instability. Difficulties for identifying this effect of myc could stem from the numerous safeguards against cancer progression and DNA damage, including myc induced apoptosis and G2 arrest [53]. It may well be that re-replication induced by myc leads to mitotic catastrophe, which is partly compensated by induction the SUMO-activating enzyme [86]. P53 is likely to play an essential role on counteracting the effect of myc, as indicated by the facts rhat its presence prevents gene amplification [87, 88], while its absence is insufficient for generating CIN [89].

In addition to myc, other oncogenes have been shown to induce genetic instability [90-92] (**Table 1**). There is evidence that cyclin D1 and ras induce overreplication [93, 94]. While the effect of cyclin D1 might be directly related to its effect on replication licensing [81], the ras protein, which is attached to the cell membrane, is more likely to act indirectly. It is therefore interesting to note that ras controls myc activity [95, 96].

Other proteins of the MCM complex have been found to induce re-replication when overexpressed (reviewed in Blow [81]), behaving as «mutators», but they are not involved in tumorigenesis, suggesting that for both processes to occur (transformation and genetic instability),

cells should also be pushed to proliferate.

Oncogene induced impairment of DNA replication as a cause of chromosomal alteration

In contrast to the mechanism described above, lack of function of various tumor suppressor genes or overexpression of cyclin E does not result in overreplication, but in extended S phase, due to impairment of S phase progression [97] (**Table 1**). Overexpression of cyclin E, as well as Retinoblastoma (Rb) protein inactivation, may also act by forcing premature S phase entry under conditions of nucleotide deficiency, which causes DSB [98]. In either case, unlike in Schimke's model, sites where chromosomal damage occur would be those where DNA has not been replicated [99]. Interestingly, c-myc was able to rescue replication-induced DNA damage, presumably by increasing the nucleotide pool [98]. This latter result, together with the fact that the consequence of oncogene action may be either an extended [97] or a shortened [100] S phase, is an indication that the major determinant of gross chromosomal alterations of tumor cells is actually unbalanced DNA replication.

Oncogene induced genetic instability: is DNA replication stress response involved?

It is often stated that cancer cell genomic instability is the consequence of a DNA replication stress [101]. This statement is ambiguous, as the term of stress itself is, since it is not clear if it is a cause or an effect. Indeed, most of the responses to DNA damage are protective, not for the cell, but for the multicellular organism. Thus, some of the responses are there to repair DNA and others are made to prevent survival or proliferation of the cell with damaged DNA. DNA damage checkpoint genes like ataxia-telangiectasia-mutated (ATM) and p53 not only oppose to DNA damage but to subversion of growth by oncogenes [54]. Limiting oncogene-induced replicative stress promotes transformation [102]. Clearly, whether it is at the DNA level or at the cell level, the stress response is a mechanism directed against genetic instability. Confusion is fed by the fact that other kinds of «stress», like oxidative stress, can lead to DNA damage. The question of oxidative stress has been discussed previously. But what actually causes DNA damage, when oncogenes are over-expressed, is either overreplicated DNA or insuf-

ficiently replicated DNA. Therefore, rather than oncogene-induced replication stress, it may be more accurate to address genomic instability as an effect of oncogene-induced unbalanced replication.

Role of oncogenes and tumor suppressor genes in whole chromosome aneuploidy

The contribution of whole chromosome aneuploidy to cancer development may be less critical [103] than mutations affecting genomic structure or sequence as also attested by the rarity of mutations involving mitotic checkpoint genes in familial cancer. Nonetheless, whole chromosome aneuploidy is part of the alterations observed in secondary genetic instability (whole chromosome instability: w-CIN). Several tumor suppressor genes with gatekeeper function have been shown to control ploidy [104] (**Table 1**). APC plays a role in kinetochore-microtubule attachment [105]. Inactivation of the tumor suppressor RB leads to elevated expression of the mitotic checkpoint gene Mad2 [106]. Mad2 overexpression uncouples cell cycle progression from mitosis, leading to aneuploidy [107]. Absence of p53 also leads to up-regulation of Mad2 [107]. All the above mentioned mechanisms involve a loss of the mitotic checkpoints [108]. Another, possibly distinct, mechanism for inducing aneuploidy is centrosome amplification [109, 110]. Oncogene activity and lack of tumor suppressor gene function may lead to supernumerary centrosomes [111, 112], but the molecular mechanisms responsible for this alteration are not fully understood. P53 may act directly at the centrosome level [113]. Furthermore, it induces tolerance to aneuploidy [114]. Unlike s-CIN, w-CIN cannot be accounted for by unbalanced DNA replication. However, here again, the relationship between genetic instability and loss of cell cycle control might be easily explained by uncoupling of the cell structure replication steps.

A general model for secondary genetic instability

In unicellular organisms, cell cycle control aims to ensure that cell components are properly replicated before the cell divides. In multicellular organisms, another level of control is required to avoid cell proliferation detrimental to the individual. There is intricacy between these two levels of control, and when a gene control-

ling cell proliferation is altered, cell components may not be homogeneously replicated. When cells are submitted to extracellular mitogenic signals, they check that they can proceed through a full cycle. In suboptimal growth conditions, cells remain in a G1 quiescent state [115]; when c-myc is overexpressed they arrest, instead, in G2 [53]. This means that myc is unable to drive homogeneous cell replication, but is able to overcome the G1/S checkpoint, probably due to its role in DNA replication. It is likely that uncontrolled c-myc induced replication results in the activation of the G2 checkpoint. Similarly, RB deficiency leads to a G2 arrest [116]. Loss of the G2 checkpoint due to further mutations may permit the cell to complete the cell cycle, but not necessarily by completely correcting replication imbalance. This remaining small imbalance may be the source of further genetic alterations, leading to cell death in most cases, but for a minority of the cell progeny, to adaptation with greater malignant potential and higher genetic instability. In the end, escaping the constraints on growth control leads to the selection of cells endowed not only with high proliferative capacity, but with genomic instability allowing their progeny to survive in a hostile environment by acquiring the various features of cancer cells. As a Darwinian mechanism, cancer produces cells adapted to survive when and where they should not. This adaptation, which explains the common hallmarks [117] of cancer cells, can be explained by a genetic instability rooted in abnormal cell replication.

Acknowledgments

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