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Tumor Hypoxia and Genetic Alterations in Sporadic Cancers

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Abstract

The cancer genome contains many gene alterations. How cancer cells acquire these alterations is a matter for discussion. One hypothesis is that cancer cells obtain mutations in genome stability genes at an early stage of tumor development, which results in genetic instability and generates a gene pool that enhances cellular proliferation and survival. Another hypothesis puts its emphasis on the natural selection of gene mutations for fitness. Recent data for systematic cancer genome sequencing shows that mutations in stability genes are rare in human sporadic cancers. Instead, many “passenger” mutations that do not drive the carcinogenesis process have been found in the cancer genome. Both the hypotheses mentioned above fall short in explaining recent data. Recently, many studies demonstrate the role of the tumor microenvironment, especially hypoxia and reoxygenation, in genetic instability. In this review, literature will be presented which supports a third hypothesis, i.e. that hypoxia/re-oxygenation induces genetic instability.

Keywords

Tumor Hypoxia; Microsatellite Instability (MSI); Chromosomal Instability (CIN); Genetic Instability and Cancer Genes

Introduction

Cancer is a disease of a cell that gains the ability to multiply in an uncontrolled way, to invade from the primary site to surrounding tissues, and to metastasize to distant sites. Throughout the past three decades, the field of cancer genetics has identified critical genes and the pathways1 whose dysfunction leads to major cancer phenotypes: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis.2 Thus, cancer is a multi-genetic disease and exhibits a progressive process where the genetic or epigenetic alterations responsible for these phenotypes accumulate in time and space.

To date, about 350 cancer genes have been identified.3 Results of recent systematic DNA sequencing of the cancer genome have show the following: 1) There are two types of mutations in cancer cells: `Driver' and `Passenger'. Driver mutations contribute to tumor cell growth and survival under restricted conditions and are positively selected during the course of cancer development. The rest of the mutations are `passenger' mutations, which have not contributed to cancer development or been positively or negatively selected. 2) Most of the mutations found in the cancer genome are passengers. 3) The frequency of mutation in each driver is low, suggesting that the number of “Drivers” in common adult sporadic cancers could be greater than the 5–7 which have been estimated by age-incidence statistics. 4)

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There is heterogeneity in the number of gene mutations among cancers; some genomes have >100,000 point mutations whereas others have less than 1000.3 This suggests that greater than 99% of mutations in the cancer genome do not contribute to carcinogenic process. How do cancer cells gain such excessive numbers of mutations?

There are three types of cancer genes; oncogenes, tumor suppressor genes and stability genes.1 Oncogenes encode proteins that promote cell multiplication and survival. Their expression or functions are activated by point gene mutation, fusion to another gene by chromosomal translocation, and gene amplification. About 90% of cancer genes are dominant acting oncogenes.3 Tumor suppressor genes encode proteins that inhibit cell multiplication and promote cell death. Inactivation of tumor suppressor genes is achieved by point mutation, gene deletion or insertion, or by epigenetic silencing. Activation of oncogenes or inactivation of tumor suppressor genes confers cell growth and gives the cancer cell a survival advantage. On the other hand, stability genes encode proteins whose loss or over-expression increases genetic alterations all over the genome. The stability genes include DNA repair genes, DNA damage sensor genes and cell cycle checkpoint genes.

A malfunction of stability genes could be the driving force of the carcinogenic process.4, 5, 6 Alternatively they may not be necessary for carcinogenesis but may merely promote this process.7 This topic is one of issues that will be discussed in this review.

Most solid tumor tissues, even when they are microscopically small, contain acute and chronic hypoxic and/or anoxic areas where oxygen pressure is lower than is physiologically normal.8, 9 As an adaptive response to the lack of oxygen, cancer cells may change their genome to increase their survival. In 1996, Glazer's group first presented evidence that the tumor microenvironment, especially hypoxia, induces high levels of gene mutations in cancer cells. This study was based on their hypothesis that “the microenvironment may give conditions that either increase DNA damage or compromise the DNA repair process”.10 Since then, this hypothesis has been tested by many research groups.11 The results of these studies generated a new concept that the microenvironment (hypoxia) induces genetic instability.12 This hypothesis accepts the idea of “genetic instability as a hallmark of cancer”, however, the extension of the hypothesis does not necessarily require the idea that cancer, especially sporadic cancer, gains gene mutations in putative stability genes that may drive carcinogenic process.4, 5, 6

If hypoxia (the microenvironment) plays a role for the carcinogenic process, it should contribute to the progression stage of tumor development when a part of the tumor tissue starts to receive less oxygen. In fact, many clinical and experimental observations indicate that hypoxia is associated with aggressiveness of tumor cells, leading to poor prognosis and metastasis in a variety of human cancers.

Within tumor tissues, oxygen concentrations fluctuate both spatially and temporally. Hypoxic tumor cells may be re-exposed by a higher concentration of oxygen (re-oxygenation), which can alter the cancer genome and contribute to tumor progression.

In this review, mechanisms by which hypoxia and re-oxygenation induce genetic alterations in sporadic cancer will be considered. Toward this goal, literature relating to tumor hypoxia, cellular pathways affected by hypoxia, types of genetic alterations and DNA repair systems affected by hypoxia and re-oxygenation has been compiled.

1. Tumor Hypoxia

1-1. Radio-resistance and hypoxia

The impact of hypoxia on human cancer in medicine was first recognized by radiologists. In the 1930's, the presence of hypoxia in solid tumor tissues was first hypothesized based on the observation that low levels of oxygen (hypoxia) protect a cell from the lethal effects of ionizing radiation and that some solid tumors are resistant to radiation.13 In 1955, Thomlinson and Gray reported histological observations of tumor cords with and without central necrosis in human lung tumors, suggesting the presence of an oxygen gradient within a tumor cord. They found that 1) all of the tumor cords surrounded by the stroma and larger than 200μm in radius contained central necrosis, 2) none of the tumor cords less than 160μm in radius contained central necrosis, and 3) no intact tumor cells were found at a distant of 180μm from the stroma. Based on these results and the calculated distance of oxygen diffusion (150μm), they proposed the presence of radio-resistant hypoxic cells at the edge of the necrotic area.14 Until the late 1980's when polarographic electrodes were used to directly measure levels of oxygen in human cancer tissues, the presence of tumor hypoxia was speculative.15, 16 During the 1990's, several key findings were made using various methods for directly detecting tumor hypoxia in human tumor tissues.9, 15 These findings are as follows: 1) Hypoxic and anoxic areas exist in most solid tumors (areas with less than 2.5 mm Hg of oxygen pressure). 2) There is no predictable association between tumor hypoxia and other clinical factors including size, stage, grade and site. 3) Tumor hypoxia may be an adverse prognostic factor.9, 17 4) Tumor hypoxia not only induces radiation-resistance but may also induces resistance to chemotherapeutic agents.9, 18

1-2. Acute and chronic hypoxia in tumor tissues

Using DNA-binding chemical Hoechst 33432, cell sorting and radiation, Chaplin et al first demonstrated that two types of hypoxia exist in solid tumor tissues.19 Because of the abnormal structure, distribution and function of microvessels formed by unregulated angiogenesis within tumor tissues, an inadequate blood flow occurs in geometrically different parts of tumor tissues. This type of hypoxia, called acute hypoxia, lasts from minutes to hours, and is followed by re-oxygenation.16, 19 Another type of hypoxia is caused by reduction of oxygen diffusion due to an increase in the distance of the tumor cells from tumor or host vasculature. This type of hypoxia is called diffusion-limited hypoxia or chronic hypoxia. It may last days followed by re-oxygenation or cell death.16 It has been suggested that a different biology may exist between acute and chronic hypoxia and this might influence interpretation of clinical and experimental data, and the design of treatments for hypoxic tumors.20

1-3. Cellular response to hypoxia

While struggling to overcome the radiation-resistance of hypoxic tumors, many aspects of the cellular response to hypoxia have been recognized and studied. These hypoxic responses are related to angiogenesis, glycolysis, metastasis, stress response, erythropoiesis and genomic stability.20, 21

1-3-1. Hypoxia-inducible factors (HIFs)—Hypoxia-inducible factors (HIFs) play a central role in these responses to hypoxia. In 1995, Wang et al. identified one of the HIFs, HIF1, a complex between HIF1α and HIFβ subunits, which is stabilized in response to hypoxia and regulates transcription of its target down-stream genes.22 HIF1 binds to the hypoxia response elements (HRE), 5'-G/ACGTG-3', in the promoter region of target genes, like *EPO*,23 *VEGF*,24 *Aldolase, Enolase,* and *LDHA*25. Currently, transcription of at least 70 known genes, and probably more, is regulated by HIFs through recognition of HRE.26 There are three HIFα family subunits, HIF1α, HIF2α and HIF3α, and they form a

heteroduplex with a common constitutive HIFβ subunit. Both the HIF1 and HIF2 heteroduplexes function as transcription factors for genes containing HRE under hypoxia. HIF1α and HIF2α but not HIFβ subunits are rapidly degraded by ubiquitin-protease pathway in normoxic conditions through oxygen-dependent degradation domain.27 A tumor suppressor protein, von Hippel-Lindau (VHL), binds to HIFα subunits and promotes oxygen-dependent degradation of HIF.28 VHL is a part of the E3 ubiquitin ligase complex and binds directly to HIFα subunits and a ubiquitinates the subunits.29 The binding between VHL and HIFα subunits is regulated through hydroxylation of a proline residue within HIFα subunits by the family of prolyl hydroxylases (PHDs or HPHs).30, 31 Because the enzyme activity of PHDs requires oxygen and iron, the lack of oxygen or iron in a cell leads to the accumulation of HIF. Another oxygen- and iron-sensitive enzyme, FIH1 (factor inhibiting HIF1), which catalyzes hydroxylation of asparagine residue on HIFα subunits, inhibits the interaction of HIFα subunits and their transcription co-activators such as p300/CREB. Hypoxia impairs FIH1 activity, which results in formation of a HIF1/CBP/p300 complex and leads to enhanced transcription of HIF target genes.32

In sustained hypoxia (chronic hypoxia), HIF activity is attenuated by the following negative feedback mechanisms: 1) HIF up-regulates CITED2 (transcription of a CBP/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain). CITED2 binds to CBP/p300 and blocks interaction between HIF and CBP/p300 and the transactivation of HIFs in hypoxic cells.33 2) HIF hydroxylase levels are up-regulated by PHD activation, leading to destruction of HIF even if O2 levels are low.34 3) Antisense RNA against HIF1α is transcribed from the HIF1 locus in an HRE-dependent manner.34

A major mechanism for a cell to adapt to hypoxia is by using the HIF pathway that activates target pathways regulating the delivery of oxygen and its utility. However, as can be seen below, HIF1 also directly or indirectly regulates the expression of other genes involved in stability of the cellular genome.

There are two other cellular signaling pathways in response to hypoxia. These include the mammalian target of rapamycin pathway (mTOR), and the endoplasmic reticulum stress pathway. Repression of mTOR and activation of the endoplasmic reticulum stress pathway by hypoxia regulates protein synthesis through inhibition of mRNA translation.35 Although there have been only a few studies reporting the involvement of these pathways in the stability of cellular genome, it is worthwhile to briefly review these pathways.

1-3-2. mTOR pathway—The mTOR is a Ser/Thr protein kinase and forms mTOR complex 1 (mTORC1) with Raptor and GβL. Raptor is a scaffolding protein that mediates interaction between mTOR kinase and its substrates to promote mTOR signaling. GβL plays a role in stabilizing mTOR and Raptor binding. When cells are under nutrient- and energy-replete conditions, the mTORC1 activates downstream proteins including ribosomal protein S6 kinase (p70S6K), eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and eukaryotic elongation factor 2 kinase (EEF2K). Phosphorylation of these proteins promotes protein synthesis, cell growth, cell proliferation and cell metabolism.35, 36

Chronic hypoxia down-regulates mTORC1 signaling through multiple pathways to maintain cellular protein synthesis levels appropriate for suboptimal conditions. Hypoxia inhibits mTORC1 signaling through the accumulation of the tuberous sclerosis protein 1 and 2 (TSC1-TSC2) complex. TSC1 stabilizes TSC2 by forming a complex with TSC2. TSC2 is a GTPase-activating (GAP) protein and regulates the Ras homolog enriched in brain (RHEB). RHEB activates mTORC1 when it is GTP-bound. Since the TSC1-TSC2 complex promotes conversion of RHEB-GTP to RHEB-GDP, this results in the cessation of mTORC1 activity. 36 Accumulation of the TSC1-TSC2 complex is achieved through competitive inhibition of

complex formations between 14-3-3 and TSC2 by DNA-damage-inducible transcript 4 (DDIT4 or REDD1). REDD1 is up-regulated by HIF1 under hypoxic conditions, binding to 14-3-3 and dissociates TSC2 from the 14-3-3/TSC2 complex.[37, 38, 39]

Hypoxia also activates the AMP-activated protein kinase (AMPK) pathway. Hypoxic cells switch respiration from the aerobic mitochondrial chain to anaerobic glycolysis to generate ATP. This results in an increase in the AMP/ATP ratio and activates AMPK activity. AMPK phosphorylates and activates GAP in TSC2 leading to inhibition of mTORC1 through a decrease in RHEB-GTP.[40]

It has been demonstrated that the Bcl2/adenvirus E1B 19-kDa interacting protein 3 (BNIP3), which is up-regulated by HIF1, interacts with RHEB and decreases the level of GTP-bound RHEB. This results in inhibition of mTORC1 activity and subsequent cessation of protein synthesis.[41] It has also been reported that the promyelocytic leukemia tumor suppressor (PML) inhibits mTORC1 by binding and transporting it to a nuclear body under hypoxia.[42]

1-3-3. Unfolded Protein Response (UPR)—The endoplasmic reticulum (ER) is a cellular organelle for protein folding and maturing. When a cell faces a number of biochemical, physiologic or pathologic environments including nutrient depletion, oxidative stress, DNA damage, energy perturbation or hypoxia, the process of protein folding and correct assembly of mature proteins is disrupted in the ER. As a result, unfolded or misfolded proteins accumulate within the ER (ER stress). In response to ER stress, the ER generates signals which alter transcriptional and translational programs that ensure the fidelity of protein folding and maturation, effectively eliminating the unfolded and misfolded proteins, and selectively allowing translation of mRNAs whose products promote the cell's survival under hypoxic conditions. This response is called Unfolded Protein Response UPR.[36, 43] Hypoxia triggers UPR by activating three ER stress sensors including the inositol-requiring protein 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK).[36, 43] The inactive forms of these three proteins are bounded by the chaperone immunoglobulin heavy chain-binding protein (BIP) and embedded in the ER membrane. Unfolded or misfolded proteins activate these sensors by binding to BIP and dissociating BIP from these sensor proteins or by directly binding to the sensors. Activated PERK phosphorylates eukaryotic initiation factor 2 subunit α (EIF2α), resulting in inhibition of global mRNA translation and selective translation of ATF4 and other hypoxia-inducible mRNAs. Activation of IRE1 results in endoribonuclease activity against X-box-binding protein 1 (XBP1) pre-mRNA and in the selective expression of XBP1. Activation of ATF6 results in its translocation to the Golgi apparatus and its cleavage to gain transcriptional activity. ATF4, XBP1 and ATF6 transactivate genes whose products increase protein folding and maturation in the ER and genes whose products remove unfolded and misfolded proteins from the ER.[36, 43]

2. Hypoxia/Re-oxygenation (H/R) and Oxidative Stress

Re-oxygenation is a component of hypoxia-induced genetic alterations. In mammalian cells, hypoxia followed by re-oxygenation increases the production of reactive oxygen species (ROS) from affected cells.[44] ROS can damage DNA, proteins, and lipids, leading to gene mutations, apoptosis and necrosis. Therefore, hypoxic cancer cells have to deal with the toxic effect of ROS. However, if cancer cells have already acquired gene mutations, for instance mutated p53, which overcome apoptosis signals triggered by H/R,[45] these cells have an increased probability of gaining additional mutations. Although ROS can generate various types of modified bases in DNA, 7, 8-dihydro-8-oxoguanine (8-oxo-G) is frequently generated.[46] For example, hypoxic human cervical cancer cells, HeLa, were placed under 1% O_2 for 24hr, produced excessive amounts of ROS at 30 min after reoxygenation.[47] This

overproduction of ROS was transient and lasted for 2 hr after re-oxygenation. Simultaneously, the same cell population generating ROS also exhibited extensive DNA damage with 8-oxoguanine.47 The 8-oxo-G:C pair, if not repaired, generates G:C>T:A or A:T>C:G transversions. These mutations are frequently found in human sporadic cancers including lung, breast, ovarian, gastric, and colon cancers.48 In *in vivo* and *in vitro* hypoxia models, an increase in transversion mutations such as G:C>T:A and A:T>G:C have been reported,10 suggesting an important carcinogenic role of ROS generated by H/R in tumor tissues.

ROS also induces DNA slippage mutations at microsatellite sequences in human cells. When human lung cancer cells carrying plasmid vector with CA repeats were treated with ROS generating chemicals, paraquat and H_2O_2, a significant increase in deletion or insertion mutations was observed within CA repeats.49 Similarly, Gasche et al showed that the frequency of microsatellite mutations (CA repeats) in transfected plasmids was increased by H_2O_2 treatment in human colon cancer cells.50 Yamada et al. examined the effect of H_2O_2 treatment on mutation frequencies of mononucleotide (A or G repeats) and di-nucleotide repeats (CA repeats) in non-cancer human diploid cell lines. They found that H_2O_2 treatment decreased the mutation frequency of mononucleotide repeats but increased the mutation frequency of di-nucleotide repeats in non-cancer diploid human cells. They speculated that ROS induces low levels of mutations in di-nucleotide repeats.51 In accordance with the effect of ROS on microsatellite loci in human cells, Chang et al reported that non-toxic levels of H_2O_2 impair mismatch repair activity,52 which leads to DNA slippage mutations at microsatellite loci (see below).

In order to faithfully transmit genetic information to a progenitor cell, the cell is equipped with mechanisms which sense DNA damage in the genome (sensor), transmit a DNA damage-signal to repair system and cell cycle machinery (signal), and target a cell for apoptosis if damage is not repaired (effector). There is some evidence that H/R activates DNA damage response.

Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) are DNA damage signal transducers. A double-strand break is recognized by sensor protein complex MRN (MRE11-RAD50-NBS1). The sensor recruits ATM, which further activates its targets CHK1/CHK2. A single stranded DNA is sensed by ATRIP (ATR interacting protein) and recruits ATR. ATR also activates CHK1/CHK2. It has been found that acute severe hypoxia (<0.02% O_2 for less than 24h) activates both ATR and ATM without DNA damage.53 It is assumed that the activation of ATR is not transducing DNA damage but directed toward maintaining replication folk stability during severe hypoxia by phosphorylating replisome components, MCM2 and MCM3.54 However, when cells are re-exposed to oxygen, reactive oxygen species (ROS) are very quickly generated and damage cellular DNA. In response to the damage, ATM is activated and phosphorylates a downstream protein, CHK2.55, 56 The activated CHK2 causes G2 cell cycle arrest through phosphorylation of Cdc25C and Cdc2.56 There is a possibility that cancer cells may propagate new genetic alterations caused by reoxygenation-induced ROS if the cells are insensitive to the G2 arrest.54

3. H/R and Genetic Instability

3-1. Genetic Instability

The concept of “Genetic Instability” was introduced to define the cancer cells' property of new mutations with each cell division. Using tissue cultured cancer cells, Lengauer and Vogelstein first demonstrated that some but not all cancer cells continuously change their chromosome numbers with each cell division.57 They termed this type of genetic instability

as Chromosome Instability (CIN). Later, CIN was extended to characterize persistent changes not only in the number of whole or a parts of chromosomes (whole chromosome instability: W-CIN) but also changes in the structure of chromosomes (amplification, deletion, and translocations: segmental chromosome instability or S-CIN) during the lifetime of cancer cells. Based on CIN observed in tissue cultures, it is assumed that the frequent occurrence of chromosomal abbreviations observed in human tumor tissues is caused by CIN mechanisms. Great progress in understanding the molecular basis of CIN has been made through the use of experimental in vitro and animal models.58 These studies have shown that W-CIN is caused by failures in the correct transmission of chromosomes into daughter cells or the spindle mitotic checkpoint.57 On the other hand, some inherited conditions, Ataxia telangiectasis, Bloom syndrome, Fanconi anaemia and Nijimegen breakage syndrome, are called chromosome instability syndromes and associated with S-CIN and a predisposition to certain types of cancer. Through identification of the genes responsible for these conditions, it is known that S-CIN is caused by mutations of the genes involved in replication, repair and S-phase checkpoints.59

Before CIN was fully understand, another type of genetic instability, microsatellite instability (MSI or MIN), had been recognized in a small fraction of cancers. MSI is characterized as an expansion or contraction of repeat units within the microsatellite locus. The origin of MSI is thought to be replication mistakes by DNA polymerase at the microsatellite followed by failed mismatch repair.60 Therefore, the main cause of MSI found in human cancers is due to inactivation of the mismatch repair system.61

Recently, an additional form of genetic instability, point mutation instability (PIN), was proposed by Loeb's lab. This is based on their DNA sequencing data that showed that cancer exhibits a 200-fold higher mutation rate than normal at the nucleotide level.62 However, the corresponding mechanism for this type of instability is not known.

3-2. Mutations in stability genes are rare in human sporadic cancers

W-CIN can be induced by disturbance of the mitotic checkpoint, a mechanism ensuring a faithful segregation of copied chromosomes to a daughter cell, or by abnormalities in spindle and centrosome functions. The experimental evidence using animal models supports this hypothesis. A partial loss of mitotic checkpoint genes including *mad2l1, mad1l1, fzr1, plk4, bub1b, bub3, bub1*, and *cenpe* cause aneuploidy in cells derived from heterozygous mice.58 Over-expression of genes including *mad2* and *hec1* also lead to CIN.58 Moreover, these mitotic checkpoint mutant mice are predisposed to various type of cancers.58

The genes responsible for the chromosome instability syndromes mentioned above are *AMT*, *BLM*, *FANC* genes, and *NBS*1 and the loss of these gene products in a cell induces S-CIN and cancer predisposition.63, 64, 65, 66 Germline mutations in *BRCA*1, *BRCA*2, *PALB*2, *RAD*50, and *BRIP*1 are found in hereditary form of breast cancers and linked to S-CIN.67 All these genes are involved in DNA damage checkpoint, cell cycle checkpoint, homologous and non-homologous recombination repair.

However, recent data from cancer genome sequencing showed that gene mutations in these CIN genes are rare in human sporadic cancers.68 Mutations in other DNA repair genes involved in nucleotide excision repair and MMR are also rare in human sporadic cancers.68

Despite the lack of mutations in stability genes, aberrant expression of stability genes has been observed in human sporadic cancers. For example, some mitotic checkpoint gene products including AURKA, AURKB MAD2L1, PLK4, BUB1B, and BUB3 are over-expressed in various types of human cancers.58 BRCA1 is down-regulated and BRCA2 is up-regulated in sporadic breast cancers.69, 70 *FANC* genes are down-regulated in head and

neck squamous cell carcinoma.71 If up- or down-regulation of stability gene products is responsible for genetic instability in sporadic tumors, it is necessary to clarify how these genes are regulated in human cancer tissues. A strong candidate for controlling the expression of stability genes in tumor tissues is tumor hypoxia/reoxygenation.11, 12

4. Evidence for H/R as cause of genetic alterations

The following is evidence that hypoxia affects the stability of the cellular genome.

4-1. Chromosomal Alterations

4-1-1. Induction of Gene Amplification and Fragile Sites—Several early studies demonstrated that the exposure of cultured mammalian cells to hypoxia followed by re-oxygenation results in DNA over-replication and gene amplification.72, 73, 74 For instance, Rice et al showed that over-replication of cellular DNA is induced by H/R, which is followed by amplification of dihydrofolate reductase gene under methotrexate selection.73 Hypoxia followed by re-oxygenation also induces fragile-sites that trigger DNA breakages and gene amplification.75 Fragile sites are chromosomal sites that show gaps and breaks after inhibition of DNA synthesis.76 They are usually associated with repetitive sequences with tri-, tetra- and dodeca-nucleotide repeats or with AT-rich repeats. These repeats form DNA secondary structures. Based on these unique sequences in fragile sites, Durkin and Glover proposed a molecular model for fragile site instability.77 In this model, first, a dissociation of DNA-unwinding by the helicase/topoisomerase complex and DNA synthesis occurs when the action of DNA polymerase is inhibited. This creates a long stretch of single stranded DNA around the fragile site. Second, AT-rich-repeats within a single strand of DNA form a hairpin structure by self annealing. This structure further causes replication fork stalling. Although most of these structures will be detected and repaired by DNA repair machinery, some forks collapse, resulting in formation of single or double stand breaks, and present themselves as gaps or breaks on metaphase chromosomes at fragile sites.77 In support of this model, Pires et al demonstrated that acute and severe hypoxia (<0.02% O_2 for less than 8 hours) blocks DNA synthesis of human cancers through inhibition of replication initiation and elongation. This blockage is due to reduction of levels of the four dNTPs that are required for DNA synthesis.54 A break at a hypoxia-induced fragile site may initiate gene amplification through the breakage-fusion-bridge mechanism.78

4-1-2. Induction of tetraploidy and sister chromatid exchange (SCE)—Another example of H/R-induced chromosomal alterations was reported by Rofstad et al.79 They examined the effects of severe hypoxia (<0.01% O_2 for 24hr) on chromosome contents of diploid as well as hyperdiploid human melanoma cell lines. They found that a subpopulation of diploid cells was arrested at the G2/M boundary during hypoxia exposure. During the first M phase after re-oxygenation, they observed a cell population which showed tetraploid chromosomes where homologous chromosomes were grouped in pairs (diplochromosomes), suggesting that severe H/R may disturb cell mitosis.79, 80

Lee et al. placed PHA-stimulated normal human lymphocytes from 40 healthy donors under mild hypoxia (3% oxygen concentration) for 12hr or 24 hr.81 After hypoxia exposure, the cells were subjected to chromosomal analysis. They found that the frequency of SCE (recombination between homologous sister chromatids) was higher in hypoxia treated cultures than normoxia cultures.80 The mechanism for SCE by H/R is not clear, however, because a perturbation of DNA synthesis results in SCE, these results suggest that even moderate levels of hypoxia followed by reoxygenation affects the DNA synthesis of a normal cell.

4-2. Increase in Gene Mutation Frequency

The effect of hypoxia on gene mutations has been examined by several mutation assay systems. Reynolds et al transplanted tumorigenic mouse cells into nude mice or placed the cells under hypoxic conditions *in vitro*.10 These cells were marked with a lambda shuttle vector containing *supF* as a reporter for mutations. The results showed a significant increase in point mutations and small deletions in DNA rescued from hypoxic cells transplanted into nude mice as well as in cells exposed to hypoxia in tissue cultures. Sixty two percent of point mutations showed transversion (G>T, G>C and A>C) and 38% were transitions (G>A) in DNA from hypoxic cells. In contrast, the percentage of transition (62%) mutations dominated over transversion mutations (38%) under normoxic conditions.10 Because the major oxidative DNA damage product, 8-oxo-G, can produce transversion mutations (G>C or G >T),46 the observed increase in mutation frequency may be caused by oxidative damage. This was supported by Keysar et al who showed that the free radical scavenger dimethyl sulfoxide blocked hypoxia-induced gene mutations.82 Because hypoxia itself does not cause DNA damage,55 oxidative stress must be generated during re-oxgenation. Similarly, Rapp-Szabo et al reported that hypoxia/re-oxygenation increased the mutation frequency of a reporter gene, *lac*I, integrated into the cellular DNA of cell lines derived from the BigBlue rat.83 They observed a small bias of transversion mutations against transition mutations in hypoxic cells in tissue cultures. These results suggest that H/R increases mutation frequency through oxidative damage and/or suppression of DNA repair like base excision repair pathways.84

4-3. Increase in Microsatellite Slippage Mutations

Three studies have demonstrated that hypoxia generates mutations within microsatellite repeat sequences in mammalian cells. Mihaylova et al transfected hypoxic HeLa and mouse EMT6 cells with an episomal reporter construct containing poly CA repeats which disrupt functional β-galactosidase by out-of frame. When slippage mutations occur within CA repeats and restore a proper reading frame, a rescued construct in bacteria can be positive for lacZ staining. The results showed a 1.6-fold increase in mutation frequency of CA repeats was induced by hypoxia (<0.001% O_2 for 48hr).85 Koshiji et al showed that the hypoxic (1% O_2 for 16hr) MLH1-deficient colon cancer cell line, HCT116, exhibits enhanced microsatellite mutations compared to normoxic cells.86 Rodriguez-Jimenez et al placed mouse neural and human mesenchymal stem cells under moderate hypoxic conditions (1% O_2) for several days. They used plasmid DNA containing out-of-frame poly (CA) repeats similar to the one used by Mihaylova et al to monitor the effect of hypoxia on microsatellite mutations. They found a significant increase in frame-sift mutations in CA repeats in the plasmid DNA. They also detected mutations in the endogenous microsatellite loci within the cellular genome in both mouse and human hypoxic stem cell cultures.87 Taken together, these observations suggest that H/R-induced microsatellite mutations are caused by repressed mismatch repair systems.85, 86, 87, 88, 89, 90 However, slippage mutations at the microsatellite locus due to loss of MMR are replication-dependent,60 and therefore it is not clear how mutations are generated when DNA synthesis is blocked by severe hypoxia (<0 0.1% O_2) as observed by Mihaylova et al.85

5. Depression of DNA Repair System by Hypoxia

Observed increases in mutation frequencies in cellular DNA could be due to altered DNA repair systems and/or increased DNA damage by H/R as discussed earlier. The following are examples of DNA repair systems modulated by hypoxia.

5-1. Homologous Recombination Repair (HRR)

When double-stranded breaks (DSB) are generated in genomic DNA during replication or by chemical or physical means, the breaks must be sealed to avoid cell death. To ensure this, cells are equipped with two types of repair systems, homologous recombination repair (HRR) and non-homologous end joining (NHEJ). HRR requires intact homologous sequences, usually sequences on a sister chromatid or a homologous chromosome, as a template for repair. It operates during the S or G2 phase of the cell cycle because of its requirement for intact sister chromatid and the availability of HRR genes. Thus, HRR is error-free. On the other hand, if HRR is deficient or damage occures at the G1 or G0 phase, cells use the alternative NHEJ pathway to repair DSBs. The NHEJ is error-prone and contributes to genetic instability. After recognition of the DSB followed by modification (resection) of a broken end through the early phase of HRR, RAD51 binds to a single stranded end and starts to look for a homologous template (invasion) and other components of HRR initiates the repair reactions.91 Recently, Bunting et al. showed the evidence that BRCA1 removes 53BP1 protein which inhibits resection through its binding to the broken ends. Because resection is an obligatory process for HRR, a removal of 53BP1 by BRCA1 initiates the HRR pathway. Thus, if BRCA1 is absent, DSBs are repaired by error-prone NHEJ.92

Bindra et al have demonstrated that *RAD*5193 and *BRCA*194, components of homologous recombination repair, are transcriptionally down-regulated by chronic hypoxia (RAD51: 0.01~ 0.5% oxygen concentration over 24 hr, BRCA1: 0.01~1% O_2 for over 24hr). This down-regulation of RAD51 and BRCA1 also reduced functional HR activity.93, 94 Furthermore, they showed that transcriptional repression of both *RAD*51 and *BRCA*1 are HIF-independent and are mediated through the binding of repressive E2F4/p130 complex at the E2F site within the promoter region of these genes.94 95 Similarly, Meng et al reported down-regulation of RAD51 in both normal and cancer cells (0.2% O_2 for 48–72hr).96

Chan et al. demonstrated that chronic hypoxia (0.2% O_2 by 72hr) down-regulates several HRR proteins including RAD51, RAD51B/C, RAD54 and XRCC3 in the human lung carcinoma cell line.97 Down-regulation of these proteins by hypoxia was associated with a decreased level of HRR activity as measured by the internal reporter system. Furthermore, they observed that decreased levels of these HRR proteins was due to reduced translation of corresponding mRNAs, suggesting involvement of the mTORC1 or UPR pathways described above. They also demonstrated that depressing HRR by chronic hypoxia increased the cells' sensitivity to the DNA cross-linking agents mitomycin C and cisplatin, and to radiation.92 Recently, a new pathway to down-regulate one of HRR gene, *RAD52*, by hypoxia was reported. Crosby et al. showed that HIF1-dependent up-regulation of the microRNAs, mir-210 and mir-373, results in suppression of *RAD52* transcription.98 They showed that both microRNAs interact with 3' untranslated region of *RAD52*, suggesting that mir-210 and mir-373 are responsible for the repression of RAD52.98 Taken together, these results suggest that depression of HRR by hypoxia may force a cell to use error-prone NHEJ that generates many genetic alterations.

5-2. Nucleotide excision repair (NER)

Yuan et al showed that hypoxia increases the UV-induced mutation rate in tissue culture cells, suggesting that hypoxia represses NER.99 Later, Crosby et al. showed that hypoxia up-regulates mir-373 which in turn degrades the RAD23B transcript, one of the genes involved in NER.98 RAD23B recognizes UV-induced DNA damage in association with XPC and this complex recruits proteins including XPA, RPA, XPB, XPD for DNA unwinding. A small patch of single stranded DNA containing damage is excised by XPG

and XPF/ERCC1, and repaired and sealed by polymerase and ligase respectively.100 Thus, repression of RAD23B by hypoxia can impair NER.

5-3. DNA Mismatch Repair (MMR)

During replication, DNA polymerase sometimes incorporates a wrong base generating a mismatch or generates a single stranded loop within a highly repetitive sequence, for instance at a microsatellite locus. These mistakes are repaired prior to mitosis by the mismatch repair (MMR) pathway. There are six MMR proteins involved in this system in humans, MSH2, MSH6, MSH3, MLH1, PMS2, and MLH3. The recognition of mismatch or loops containing one nucleotide is mainly mediated by MutSα (a heterodimer of MSH2 and MSH6). Recognition of a loop containing two or more nucleotides is mediated by MutSβ. 101 Excision of a mismatched base or a loop on a newly synthesized strand is initiated by recruited MutLα (a heterodimer of MLH1 and PMS2) or MutLβ (a heterodimer of MLH1 and MLH3) and followed by exonuclease (EXO1). Re-synthesis is done by DNA polymerase and the nick is sealed by DNA ligase.101 If one of six MMR proteins is disabled, the mutation frequency in the microsatellite sequences increases.

The microsatellite slippage mutations described above are associated with hypoxia-induced repression of MMR proteins including MSH2, MSH6, MSH3, MLH1 and PMS2.85, 86, 87 Mihaylova et al first demonstrated that severe chronic hypoxia (<0.001% O_2 by 48hy) down-regulates MLH1 at the transcriptional level in several mouse and human cancer cell lines. This effect is blocked by the histone deacetylase inhibitor, trichostatin A, suggesting that down-regulation may be caused by histone deacetylation at the *hMLH*1 locus.85 Koshiji et al. reported that hypoxia (1% O_2 for 16 hr) down-regulates transcription of MSH2 and MSH6 in the MLH1-negative cell line, HCT116.86 This effect is p53-dependent and HIF1-dependent. They demonstrated that transcriptional repression of MSH2 and MSH6 by hypoxia is mediated by reduction of the Sp1-MYC complex which promotes MSH2/MSH6 transcription under normoxic conditions. Because HIF1 competes with MYC in forming a complex with Sp1, stabilization of HIF1 by hypoxia results in the reduction of the Sp1-MYC complex.86 Koshiji's work was followed by that of Bindra and Glazer, who demonstrated that both MSH2 and MLH1 are transcriptionally down-regulated by prolonged severe hypoxia (0.01% O_2 for 48hr) in human cancer cell lines from different tissues and in normal human cell lines.102 In contrast to Koshiji's work, they observed a correlation between down-regulation of *MYC and MSH2/MLH1* transcriptions in hypoxic cells. They found that the occupancies of both MSH2 and MLH1 promoters by MYC were replaced by MAX, MAD1 and MNT in hypoxic cells. They also demonstrated that down-regulation of *MSH2/MLH1* is HIF-independent. Based on these results they proposed the model that repression of MSH2/MLH1 by hypoxia is mediated through a HIF-independent, MYC/MAX network. 102 The discrepancy between Koshiji's and Bindra's studies might be explained by the difference in oxygen concentrations they used (1% versus 0.01% respectively). Interestingly, however, Shahrzad et al showed that no significant decrease in MSH2 protein level was observed in HCT116 under hypoxic conditions (<0.1% O_2 for 24 hr).90 These results suggest that expression of MMR genes may be differentially controlled by different mechanisms according to the concentration of oxygen and duration of hypoxia. In support of this notion, Nakamura et al have showed that the gene products of HIF1 inducible genes, DEC1 and DEC2 (differentiated embryo chondrocytes 1 and 2), down-regulate transcription of the *MLH1* through the repressor functions of these proteins.89 They observed down-regulation of MLH1 at mRNA and protein levels in hypoxic cells (1% O_2 for 6, 12, 24, 48 or 72 hr). This down-regulation is associated with up-regulation of DEC1 & 2. They found DEC1 & 2 binding sites (E-box) within the *MLH1* promoter region, and that the binding of DEC1 & 2 to the sites represses the promoter activity of MLH1. They further showed that silencing of HIF1 or DEC2 by corresponding siRNAs in hypoxic cells canceled down-

regulation of MLH1. Base on these results, they concluded that down-regulation of MLH1 by hypoxia is mediated by a HIF1-dependent increase of DEC1 and DEC2 proteins.89 Rodriguez-Jimenez et al demonstrated that MMR including MLH1, MSH2, MSH6 and MSH3 are down-regulated at mRNA and protein levels by hypoxia (1% O_2 up to 48hr) in mouse neural and human mesenchymal stem cells.87 They showed evidence that down-regulation in the *MSH6* and *MLH1* loci is HIF-independent, and associated with Sp1 binding regulated by histone deacetylation.87

Although many different mechanisms are proposed for repression of MMR genes, these studies support that hypoxia represses the MMR system which leads to an increase in displace and frame-shift mutations. For example, intra-tumoral heterogeneity in expression of MSH3 protein is associated with low levels of microsatellite instability in sporadic colorectal cancers, which can be explained by local hypoxia.103 It is worth mentioning that the frequencies of insertion and deletion mutations, which may be mediated by repression of the MMR system, are high in sporadic cancers including breast, lung, stomach and ovary.48

6. Other hypoxia targets relate to DNA transactions

6-1. Up-regulation of mitotic spindle checkpoint gene expression by hypoxia

As discussed earlier, mutations in mitotic spindle check point genes are rare in sporadic human cancers. However, the abnormal expression of these genes is widely spread among variety of human cancers.58 It is possible that hypoxia may alter the expression of mitotic spindle genes and trigger CIN phenotype in cancer cells. For example, the mitotic spindle checkpoint gene, *AURKA (STK15)*, regulates chromosome segregation during mitosis. Its over expression results in centrosome amplification and leads to CIN. Over-expression of *AURKA* is found in breast, colorectal, ovarian, pancreatic, gastric, oesophageal, bladder, cervical and head and neck cancers.58 Klein et al demonstrated that hypoxia (3% O_2) quickly up-regulates *AURKA* at the mRNA and protein levels in hepatocellular carcinoma cells. This up-regulation is HIF1-dependent and mediated by binding of HIF1 at the HRE site.104 It would be interesting to determine whether expressions of other mitotic spindle genes including AURKB, BUB1B, BUB3, CDC20, FZR1, CENPE, CCNB1, NDC80, MAD1L1, MAD2L1, PTTG1, PLK1 and PLK4 are controlled by hypoxia through the HIF1-pathway because these genes contain putative HRE sites (5'- G/ACGTG-3') within a 5' promoter region.

6-2. Up-regulation of error-prone DNA polymerase ι by hypoxia

A replication fork stalls when it encounters DNA lesions. Prolonged stalling results in the corruption of the replication fork, leading to cell death. Pol ι is one of several DNA polymerases involved in translesion synthesis.105 These polymerases replicate a template regardless of the presence or absence of DNA damage, thus bypassing the lesions. Ito et al demonstrated that hypoxia (1% O2 for 6hr) up-regulates Pol ι at mRNA and protein levels in cancer cells.47 They also identified a functional HRE element within intron1 of the Pol ι gene, suggesting that up-regulation of Pol ι by hypoxia is HIF1-dependent.47 Among ROS generated during H/R, the hydroxy radical (OH^-) can cleave the bases from DNA and generate simple apurinic/apyrimidinic sites. Pol ι can bypass AP sites efficiently but also misincorporate bases at the site because of its low fidelity.106 It may be that at the expense of generating mutations, mammalian cells may use transient up-regulation of Pol ι to deal with replication arrest by DNA damage for survival.107 However, continuous over-expression of such error prone DNA polymerase, for instance by chronic hypoxia, may result in a high rate of point mutations.108

6-3. Down-regulation of NBS1 expression by hypoxia

As mentioned above, germline mutations in *NBS1* predispose to the Nijimegen breakage syndrome. The NBS1 protein forms a complex with MRE11A and RAD50 called MRN. MRN interacts with double strand breaks and begins the DNA damage response by recruiting the ATM protein (see above). Inactivation of NBS1 impairs the function of MRN, leading to a high sensitivity to radiation, CIN, and defective cell cycle checkpoints. To et al. demonstrated that hypoxia (1% O_2 for 16 hr) down-regulates NBS1 expression at the mRNA and protein levels in cancer cell lines.109 They showed that this down-regulation is HIF1 but not HIF2 dependent and is mediated by reduction of Sp1-MYC by competing Sp1-HIF1 at the promoter region of the *NBS1* locus, similar to the *MSH2* locus.86, 109

7. H/R-associated Genetic Instability

All cancers contain a much greater number of genetic and epigenetic alterations than do corresponding normal cells. At nucleotide levels, these alterations include: substitutions of one base by another, insertions or deletions of small or large segments of DNA, rearrangements, copy number increases, copy number reductions, acquisition of foreign DNA (virus) in some cases and hypermethylation or hypomethylation of guanosine residue. 3 The cancer genome also shows changes in numbers of whole or parts of chromosome.

It is reasonable to assume that these genetic alterations can be caused in part by exposure to environmental carcinogens. Data from the whole genome sequencing of melanoma showed a clear contribution of UV-radiation to the melanoma genome.110 Interestingly, a sign of the second genetic insult after UV damage is detected in the genome and this is characterized by an increase in the frequency of C>A transversions.110 It is tempting to speculate that the second event occurred in the melanoma genome may be associated with H/R.

As reviewed in this article, H/R is a strong candidate for induction of genetic alterations and the DNA damage response found in cancer genomes and tissues. However, our insights into H/R on the cellular genome are all based on experiments performed in tissue culture or in animal models. The question is whether H/R really plays the same contributing role for genetic instability in human tumor tissues as observed in experimental systems.

If H/R-induced genetic instability is in fact operative in human cancer tissues, we can predict some cancer genotypes. 1) Except for the main “Driver” which allows their progenitor cell to expand, genetic alterations must be highly heterogeneous within a tumor tissue due to the geographical and oxygen pressure heterogeneity of hypoxia. 2) Many types of genetic alterations should be seen in a single tumor cell. 3) Mutations in stability genes might be very rare because they are not necessary.

We know that H/R is associated with a poor prognosis, metastasis, and radio- and chemo-resistance in a variety of human cancers.20 H/R can generate a mutated gene pool and set the field to select genes responsible for worse phenotypes. Managing tumor hypoxia may be an effective way to treat cancers.111

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References

1. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nat Med. 2004; 10:789–799. [PubMed: 15286780]
2. Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell. 2000; 100:57–70. [PubMed: 10647931]
3. Stratton MR, Campbell PJ, Futreal PA. The Cancer Genome. Nature. 2009; 458:719–724. [PubMed: 19360079]
4. Loeb LA. Mutator phenotype may be required for multistage carcinogenesis. Cancer Res. 1991; 51:3075–3079. [PubMed: 2039987]
5. Loeb LA, Loeb KR, Anderson JP. Multiple mutations and cancer. Proc Natl Acad Sci USA. 2003; 100:776–781. [PubMed: 12552134]
6. Rajagopalan H, Nowak MA, Vogelstein B, Lengauer C. The significance of unstable chromosomes in colorectal cancer. Nat Rev Cancer. 2003; 3:695–699. [PubMed: 12951588]
7. Sieber OM, Heinimann K, Tomlinson IP. Genomic instability – the engine of tumorgenesis? Nat Rev Cancer. 2003; 3:701–708. [PubMed: 12951589]
8. Moulder JE, Rockwell S. Tumor hypoxia: its impact on cancer therapy. Cancer Metastasis Rev. 1987; 5:313–341. [PubMed: 3552280]
9. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst. 2001; 93:266–276. [PubMed: 11181773]
10. Reynolds TY, Rockwell S, Glazer PM. Genetic instability induced by the tumor microenvironment. Cancer Res. 1996; 56:5754–5757. [PubMed: 8971187]
11. Bindra RS, Crosby ME, Glazer PM. Regulation of DNA repair in hypoxic cancer cells. Cancer Metastasis Rev. 2007; 26:249–260. [PubMed: 17415527]
12. Huang LE, Bindra RS, Glazer PM, Harris AL. Hypoxia-induced genetic instability –a calculated mechanism underlying tumor progression. J Mol Med. 2007; 85:139–148. [PubMed: 17180667]
13. Mottram JC. A factor of importance in the radio sensitivity of tumours. Br J Radiol. 1936:606–614.
14. Thomlinson RH, Gray LH. The histological structure of some human lung cancers and the possible implications for radio-therapy. Br J Cancer. 1955:539–549. [PubMed: 13304213]
15. Gatenby RA, Kessler HB, Rosenblum JS, et al. Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. Int J Radiat Oncol Biol Phys. 1988; 14:831–838. [PubMed: 3360652]
16. Vaupel P, Kelleher DK, Hockel M. Oxygenation status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. Semin Oncol. 2001; 28:29–35. [PubMed: 11395850]
17. Hockel M, Schlenger K, Aral B, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res. 1996; 56:4509–4515. [PubMed: 8813149]
18. Teicher BA, Holden SA, Al-Achi A, Herman TS. Classification of antineoplastic treatments by their differential toxicity toward putative oxygenated and hypoxic tumor subpopulations *in Vivo* in the FSaIIC murine fibrosarcoma. Cancer Res. 1990; 50:3339–3344. [PubMed: 2334928]
19. Chaplin DJ, Durand RE, Olive PL. Acute hypoxia in tumors: implications for modifiers of radiation effects. Int J Radiat Oncol Biol Phys. 1986; 12:1279–1282. [PubMed: 3759546]
20. Bristow RG, Hill RP. Hypoxia, DNA repair and genetic instability. Nat Rev Cancer. 2008; 8:180–192. [PubMed: 18273037]
21. Bertout JA, PLatel SA, Simon MC. The impact of O_2 availability on human cancer. Nat Rev Cancer. 2008; 8:967–975. [PubMed: 18987634]
22. Wang GL, Jiang B-H, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA. 1995; 92:5510–5514. [PubMed: 7539918]
23. Jiang B-H, Rue E, Wang GL, Roe R, Semenza GL. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem. 1996; 271:17771–17778. [PubMed: 8663540]

24. Forsythe JA, Jiang B-H, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol. 1996; 16:4604–4613. [PubMed: 8756616]
25. Semenza GL, Jiang B-H, Leung SW, et al. Hypoxia response elements in the aldolase A, Enolase 1 and Lactate Dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem. 1996; 271:32529–32537. [PubMed: 8955077]
26. Wenger RH, Stiehl DP, Camenisch G. Integration of oxygen signaling at the consensus HRE. Sci STKE. 2005:1–13.
27. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA. 1998; 95:7987–7992. [PubMed: 9653127]
28. Maxwell PH, Wiesener MS, Chang GW, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 1999; 399:271–275. [PubMed: 10353251]
29. Ohh M, Park CW, Ivan M, et al. Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel-Lindau protein. Nat Cell Biol. 2000; 2:423–427. [PubMed: 10878807]
30. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science. 2001; 292:468–471. [PubMed: 11292861]
31. Ivan M, Kondo K, Yang H, et al. HIFα targeted for VHL-mediated destruction by poline hydroxylation: implications for O_2 sensing. Science. 2001; 292:464–468. [PubMed: 11292862]
32. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. 2002; 16:1466–1471. [PubMed: 12080085]
33. Bhattacharya S, Michels CL, Leung MK, Arany ZP, Kung AL, Livingston DM. Functional role of p35srj, a novel p300/CBP binding protein during transactivation by HIF-1. Genes Dev. 1999; 13:64–75. [PubMed: 9887100]
34. Webb JD, Coleman ML, Pugh CW. Hypoxia, hypoxia-inducible factors (HIF), HIF hydroxylases and oxygen sensing. Cell. Mol. Life Sci. 2009; 66:3539–3554. [PubMed: 19756382]
35. Foster KG, Fingar DC. mTOR: Conducting the cellular signaling symphony. J Biol Chem. 2010 http://www.jbc.org/cgi/doi/10.1074/jbc.R109.094003.
36. Wouters BG, Koritzinsky M. Hypoxia signaling through mTOR amd the unfolded protein response in cancer. Nat Rev Cancer. 2008; 8:851–864. [PubMed: 18846101]
37. Brugarolas J, Lei K, Hurley RL, et al. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev. 2004; 18:2893–2904. [PubMed: 15545625]
38. Sofer A, Lei K, Johannessen CM, Ellisen LW. Regulation of mTOR and cell growth in response to energy stress by REDD1. Mol Cell Biol. 2005; 25:5834–5845. [PubMed: 15988001]
39. DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. Genes Dev. 2008; 22:239–251. [PubMed: 18198340]
40. Liu L, Cash TP, Jones RG, Keith B, Thompson CB, Simon MC. Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol Cell. 2006; 21:521–531. [PubMed: 16483933]
41. Li Y, Wang Y, Kim E, et al. Bnip3 mediates the hypoxia-induced inhibition on mammalian target of rapamycin by interacting with Rheb. J Biol Chem. 2007; 282:35803–35813. [PubMed: 17928295]
42. Bernardi R, Guernah I, Jin D, et al. PML inhibits HIF-1α translation and neoangiogenesis through repression of mTOR. Nature. 2006; 442:779–785. [PubMed: 16915281]
43. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol. 2007; 8:519–529. [PubMed: 17565364]
44. Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am J Physiol Cell Physiol. 2002; 282:C227–C241. [PubMed: 11788333]
45. Thompson KE, Royds JA. Hypoxia and reoxygenation: a pressure for mutant p53 cell selection and tumour progression. Bull Math Biol. 1999; 61:759–778. [PubMed: 17883223]

46. Sekiguchi M, Tsuzuki T. Oxidative nucleotide damage: consequences and prevention. Oncogene. 2002; 21:8895–8904. [PubMed: 12483507]
47. Ito A, Koshikawa N, Mochizuki S, Omura K, Takenaga K. Hypoxia-inducible factor-1 mediates the expression of DNA polymerase ι in human tumor cells. Biochem Biophys Res Commun. 2006; 351:306–311. [PubMed: 17056006]
48. Greenman C, Stephens P, Smith R, et al. Patterns of somatic mutation in human cancer genomes. Nature. 2007; 446:153–158. [PubMed: 17344846]
49. Zienolddiny S, Ryberg D, Haugen A. Induction of microsatellite mutations by oxidative agents in human lung cancer cell lines. Carcinogenesis. 2000; 121:1521–1526. [PubMed: 10910953]
50. Gasche C, Chang CL, Rhees J, Goel A, Boland CR. Oxidative stress increases frameshift mutations in human colorectal cancer cells. Cancer Res. 2001; 61:7444–7448. [PubMed: 11606378]
51. Yamada NA, Parker JM, Farber RA. Mutation frequency analysis of mononucleotide and dinucleotide repeats after oxidative stress. Environ Mol Mutagen. 2003; 42:75–84. [PubMed: 12929119]
52. Chang CL, Marra G, Chauhan DP, et al. Oxidative stress inactivates the human DNA mismatch repair system. Am J Physiol Cell Physiol. 2002; 283:C148–154. [PubMed: 12055083]
53. Bencokova Z, Kaufmann MR, Pires IM, Lecane PS, Hammond EM. ATM activation and signaling under hypoxic conditions. Mol Cell Biol. 2009; 29:526–537. [PubMed: 18981219]
54. Pires IM, Bencokova Z, Milani M, et al. Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. Cancer Res. 2010; 70:925–935. [PubMed: 20103649]
55. Hammond EM, Dorie MJ, Giaccia AJ. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. J Biol Chem. 2003; 278:12207–12213. [PubMed: 12519769]
56. Freiberg RA, Hammond EM, Dorie MJ, Welford SM, Giaccia AJ. DNA damage during reoxygenation elicits a Chk2-dependent checkpoint response. Mol Cell Biol. 2006; 26:1598–1609. [PubMed: 16478982]
57. Langauer C, Kinzler KW, Vogelstein B. Genetic instability in colorectqal cancers. Nature. 1997; 386:623–627. [PubMed: 9121588]
58. Schvartzman J-M, Sotillo R, Benezra R. Mitotic chromosomal instability and cancer: mouse modeling of the human disease. Nat Rev Cancer. 2010; 10:102–113. [PubMed: 20094045]
59. Aguilera A, Gomez-Gonzalez B. Genome instability: a mechanistic view of its causes and consequences. Nat Rev Genet. 2008; 9:204–217. [PubMed: 18227811]
60. Umar A, Kunkel TA. DNA-replication fidelity, mismatch repair and genome instability in cancer cells. Eur J Biochem. 1996; 238:297–307. [PubMed: 8681938]
61. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998; 58:5248–5257. [PubMed: 9823339]
62. Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA. Human cancers express a mutator phenotype. Proc Natl Acad Sci USA. 2006; 103:18238–18242. [PubMed: 17108085]
63. Shen KC, Heng H, Wang Y, et al. ATM and p21 cooperate to suppress aneuploidy and subsequent tumor development. Cancer Res. 2005; 65:8747–8753. [PubMed: 16204044]
64. Chester N, Babbe H, Pinkas J, Manning C, Leder P. Mutation of the murine bloom's syndrome gene produces global genome destabilization. Mol Cell Biol. 2006; 26:6713–6726. [PubMed: 16914751]
65. Taniguchi T, D'Andrea AD. Molecular pathogenesis of Fanconi anemia: recent progress. Blood. 2006; 107:4223–4233. [PubMed: 16493006]
66. Shimada M, Sagae R, Kobayashi J, Habu T, Komatsu. Inactivation of the Nijmegen breakage syndrome gene leads to excess centrosome duplication via the ATR/BRCA1 pathway. Cancer Res. 2009; 69:1768–1775. [PubMed: 19244116]
67. Walsh T, King MC. Ten genes for inherited breast cancer. Cancer Cell. 2007; 11:103–105. [PubMed: 17292821]

68. Negrini S, Gorgoulis VG, Halazonetis TD. Genomic instability – an evolving hallmark of cancer. Nat Rev Mol Cell Biol. 2010; 11:220–228. [PubMed: 20177397]
69. Thompson ME, Jensen RA, Obermiller PS, Page DL, Holt JT. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet. 1995; 9:444–450. [PubMed: 7795653]
70. Bieche I, Nogues C, Lidereau R. Overexpression of BRCA2 gene in sporadic breast tumours. Oncogene. 1999; 18:5232–5238. [PubMed: 10498873]
71. Wreesmann VB, Estilo C, Eisele DW, Singh B, Wang SJ. Downregulation of Fanconi anemia genes in sporadic head and neck squamous cell carcinoma. J Otorhinolaryngol Relat Spec. 2007; 69:218–225.
72. Rice GC, Spiro IJ, Ling CC. Detection of S-phase overreplication following chronic hypoxia using a monoclonal anti-BrdUrd. Int J Radiat Oncol Biol Phys. 1985; 11:1817–1822. [PubMed: 4044344]
73. Rice GC, Hoy C, Schimke RT. Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. Proc Natl Acad Sci USA. 1986; 83:5978–5982. [PubMed: 3461470]
74. Young SD, Marshall RS, Hill RP. Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci USA. 1988; 85:9533–9537. [PubMed: 3200838]
75. Coquelle A, Toledo F, Stern S, Bieth A, Debatisse M. A new role for hypoxia in tumor progression: induction of fragile site triggering genomic rearrangements and formation of complex DMs and HSRs. Mol Cell. 1998; 2:259–265. [PubMed: 9734364]
76. Sutherland G. Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. Science. 1977; 197:265–266. [PubMed: 877551]
77. Durkin SG, Glover TW. Chromosome fragile sites. Annu Rev Genet. 2007; 41:169–192. [PubMed: 17608616]
78. Ciullo M, Debily M-A, RAozier L, et al. Initiation of the breakage-fusion-bridge mechanism through common fragile site activation in human breast cancer cells: the model of PIP gene duplication from a break at FRA7I. Hum Mol Genet. 2002; 11:2887–2894. [PubMed: 12393800]
79. Rofstad EK, Johnsen NM, Lyng H. Hypoxia-induced tetraploidisation of a diploid human melanoma cell line *in vitro*. Br J Cancer Suppl. 1996:S136–S139. [PubMed: 8763866]
80. Yih L-H, Lee T-C. Induction of C-anaphase and diplochromosome through dysregulation of spindle assembly checkpoint by sodium arsenite in human fibroblasts. Cancer Res. 2003; 63:6680–6688. [PubMed: 14583462]
81. Lee J-H, Choi I-J, Song D-K. Genetic instability in the human lymphocyte exposed to hypoxia. Cancer Genet Cytogenet. 2010; 196:83–88. [PubMed: 19963140]
82. Keysar SB, Trncic N, LaRue SM, Fox MH. Hypoxia/reoxygenation-induced mutations in mammalian cells detected by the flow cytometry mutation assay and characterized by mutant spectrum. Radiat Res. 2010; 173:21–26. [PubMed: 20041756]
83. Papp-Szabo E, Josephy PD, Coomber BL. Microenvironmental influences on mutagenesis in mammary epithelial cells. Int. J. Cancer. 2005; 116:679–685. [PubMed: 15849743]
84. Maynard S, Schuman HS, Harboe C, de Souza-Pinto NC, Bohr VA. Base excision repair of oxidative DNA damage and association with cancer and aging. Carcinogenesis. 2009; 30:2–10. [PubMed: 18978338]
85. Mihaylova VT, Bindra RS, Yuan J, et al. Decreased expression of the DNA mismatch Repair Gene *Mlh1* under Hypoxic Stress in Mammalian Cells. Mol Cell Biol. 2003; 23:3265–3273. [PubMed: 12697826]
86. Koshiji M, To KK, Hammer S, et al. HIF-1α induces genetic instability by transcriptionally downregulating MutSα expression. Mol Cell. 2005; 17:793–803. [PubMed: 15780936]
87. Rodriguez-Jimenez FJ, Moreno-Manzano V, Lucas-Dominguez R, Sanchez-Puelles JM. Hypoxia causes downregulation of mismatch repair system and genomic instability in stem cells. Stem Cells. 2008; 26:2052–2062. [PubMed: 18511603]

88. Francia G, Green SK, Bocci G, et al. Down-regulation of DNA mismatch repair proteins in human and murine tumor spheroids: implications for multicellular resistance to alkylating agents. Mol Cancer Ther. 2005; 4:1484–1494. [PubMed: 16227397]

89. Nakamura H, Tanimoto K, Hiyama K, et al. Human mismatch repair gene, *MLH1*, is transcriptionally repressed by the hypoxia-inducible transcription factors, DEC1 and DEC2. Oncogene. 2008; 27:4200–4209. [PubMed: 18345027]

90. Shahrzad S, Quayle L, Stone C, et al. Ischemia-induced K-ras mutations in human colorectal cancer cells: role of microenvironmental regulation of MSH2 expression. Cancer Res. 2005; 65:8134–8141. [PubMed: 16166287]

91. Moynahan ME, Jasin M. Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. Nat Rev Mol Cell Biol. 2010; 11:196–207. [PubMed: 20177395]

92. Bunting SF, Callen E, Wong N, et al. 53BP1 inhibits homologous recombination in *Brca1*-deficient cells by blocking resection of DNA breaks. Cell. 2010; 141:243–254. [PubMed: 20362325]

93. Bindra RS, Schaffer PJ, Meng A, et al. Down-regulation of RAd51 and decreased homologous recombination in hypoxic cancer cells. Mol Cell Biol. 2004; 24:8504–8518. [PubMed: 15367671]

94. Bindra RS, Gibson SL, Meng A, et al. Hypoxia-induced down-regulation of *BRCA1* expression by E2Fs. Cancer Res. 2005; 65:11597–11604. [PubMed: 16357170]

95. Bindra RS, Glazer PM. Repression of *RAD51* gene expression by E2F4/p130 complexes in hypoxia. Oncogene. 2007; 26:2048–2057. [PubMed: 17001309]

96. Meng AX, Jalali F, Cuddihy A, et al. Hypoxia down-regulates DNA double strand break repair gene expression in prostate cancer cells. Radiother Oncol. 2005; 76:168–176. [PubMed: 16026872]

97. Chan N, Koritzinsky M, Zhao H, et al. Chronic hypoxia decreases synthesis of homologous recombination proteins to offset chemoresistance and radioresistance. Cancer Res. 2008; 68:605–612. [PubMed: 18199558]

98. Crosby ME, Kulshreshtha R, Ivan M, et al. MicroRNA regulation of DNA repair gene expression in hypoxic stress. Cancer Res. 2009; 69:1221–1229. [PubMed: 19141645]

99. Yuan J, Narayanan L, Rockwell S, Glazer PM. Diminished DNA repair and elevated mutagenesis in mammalian cells exposed to hypoxia and low pH. Cancer Res. 2000; 60:4372–4376. [PubMed: 10969780]

100. Dantuma NP, Heinen C, Hoogstraten D. The ubiquitin receptor Rad23: At the crossroads of nucleotide excision repair and proteasomal degradation. DNA Repair. 2009; 8:449–460. [PubMed: 19223247]

101. Boland CR, Koi M, Chang DK, Carethers JM. The biochemical basis of microsatellite instability and abnormal immunohistochemistry and clinical behavior in Lynch Syndrome: from bench to bedside. Familial Cancer. 2008; 7:41–52. [PubMed: 17636426]

102. Bindra RS, Glazer PM. Co-repression of mismatch repair gene expression by hypoxia in cancer cells: role of the Myc/Max network. Cancer Lett. 2007; 262:93–103. [PubMed: 17275176]

103. Haugen A, Goel A, Yamada K, et al. Genetic instability caused by loss of MutS homologue 3 in human colorectal cancer. Cancer Res. 2008; 68:8465–8472. [PubMed: 18922920]

104. Klein A, Flugel D, Kietzmann T. Transcriptional regulation of serine/threonine kinase-15 (STK15) expression by hopoxia and HIF-1. Mol Biol Cell. 2008; 19:3667–3675. [PubMed: 18562694]

105. McDonald JP, Tissier A, Frank EG, Iwai S, Hanaoka F, Woodgate R. DNA polymerase iota and related Rad30-like enzymes. Philos Trans R Soc Lond B Biol Sci. 2001; 356:53–60. [PubMed: 11205331]

106. Zhang Y, Yuan F, Wu X, Taylor JS, Wang Z. Response of human DNA polymerase ι to DNA lesions. Nucleic Acids Res. 2001; 29:928–935. [PubMed: 11160925]

107. Budzowska M, Kanaar R. Mechanisms of dealing with DNA damage-induced replication problems. CellBiochem Biophys. 2009; 53:17–31.

108. Yang J, Chen Z, Liu Y, Hickey RJ, Malkas LH. Altered DNA polymerase ι expression in breast cancer cells leads to a reduction in DNA replication fidelity and a higher rate of mutagenesis. Cancer Res. 2004; 64:5597–5607. [PubMed: 15313897]

109. To KK, Sedelnikova OA, Samons M, Bonner WM, Huang LE. The phosphorylation status of PAS-B distinguishes HIF-1α from HIF-2α in *NBS1* repression. EMBO J. 2006; 25:4784–4794. [PubMed: 17024177]

110. Pleasance ED, Cheetham RK, Stephens PJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. Nature. 2010; 463:191–197. [PubMed: 20016485]

111. Galluzzo M, Pennacchietti S, Rosano S, Comoglio PM, Michieli P. Prevention of hypoxia by myoglobin expression in human tumor cells promotes differentiation and inhibits metastasis. J Clin Invest. 2009; 119:865–875. [PubMed: 19307731]