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Non-heme dioxygenases in tumor hypoxia: they're all bound with the same fate

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Running title: inhibition of non-heme dioxygenases during tumor hypoxia

Highlights:

- Human genome encoded several non-heme dioxygenases that require molecular oxygen for their activity.
- There is ample evidence to suggest that activity of human DNA repair proteins ALKBH2 and ALKBH3, which also belong to non-heme dioxygenase family, is inhibited during hypoxia.
- Inhibition of ALKBH2 and ALKBH3 activity could be among multiple factors that contribute towards manifestation of hypoxia-associated genomic instability.

Abstract: Tumor tissues are known to harbor hypoxic areas. The hypoxic microenvironment promotes angiogenesis. Hypoxic tumor cells also manifest genome instability. DNA damage repair pathways, such as double-strand break repair, mismatch repair and base excision repair are known to be altered during hypoxia. This review is focused on the non-heme Fe(II) and 2-oxoglutarate-dependent dioxygenases which are involved in repair of DNA alkylation adducts. Activities of these DNA repair enzymes are completely oxygen-dependent and little information is available about inhibition of these enzymes during hypoxia. While impairment of function of non-heme dioxygenase during tumor hypoxia has been implicated in different studies, the possible outcomes with respect to mutagenesis and genomic instability are explored here.

Keywords: Fe(II)/2-oxoglutarate-dependent dioxygenase; non-heme dioxygenase; Hypoxia; cancer; tumor; DNA repair; 1meA; 3meC; AlkB; *ALKBH2*; *ALKBH3*

Introduction: Mammalian cells undergo apoptotic cell death in complete lack of oxygen (anoxia). However, poor oxygenation (hypoxia) of various degrees (0.2 to 5% O_2) does not induce cell death as the cells can adapt by activating pro-angiogenic genes to regain oxygen supply. This hypoxia-adaptation process is regulated largely by the hypoxia-inducible factor (HIF) family of transcription factors. Three HIFα isoforms (HIF-1α, HIF-2α, HIF-3α) and two HIFβ isoforms (HIF-1β and HIF-2β) have been described. HIF-1α mRNA is constitutively expressed in majority of cells while the expression of HIF-2α and HIF-3α is tissue-specific. HIF-1α become stabilized under conditions of hypoxia and subsequently induces numerous genes whose products are involved in angiogenesis, metabolic adaptation to anaerobic glycolysis, cell migration and invasion [1].

Over the past three decades, it has been established that the majority of solid tumors contain regions that are hypoxic. Tumor hypoxia has been linked to poor prognosis in the treatment of several cancer types [2, 3]. Several clinical studies revealed that multiple episodes of hypoxia (0.2-5% O_2) is inherent to aggressive tumor phenotype and metastases [4]. One important factor that fundamentally alter the tumor physiology during hypoxia is manifestation of genomic instability or a mutator phenotype [5]. At molecular level, hypoxia-

induced genomic instability arise perhaps due to increased DNA damage and inhibition of some of the DNA repair pathways. The current review focuses on non-heme Fe(II) and 2-oxoglutarate (2OG)-dependent dioxygenases and discusses their possible roles in hypoxia-induced mutator phenotype and genomic instability.

Defective expression of DNA repair genes promotes genomic instability in hypoxia: One of the earlier evidence of downregulation of DNA repair genes was obtained from a large-scale microarray-based screen aimed at identifying alteration of gene expression in response to chronic hypoxia. It was revealed that under prolonged exposure to hypoxia (24h at 0.5% O_2) more than 50 DNA repair genes were transcriptionally downregulated [6]. Among them were the genes involved in homologous recombination (HR), and mismatch repair (MMR) pathways. Perhaps the only exception was nucleotide excision repair (NER) genes whose level of expression increased during hypoxia [6-9]. This could be due to the presence of a core DNA motif (G/ACGTG), known as HIF-1α response element (HRE), in several NER genes (XPC, XPD, XPB, XPG, CSA and CSB) that mediates sequence-specific DNA binding to HIF-1α and increased level of expression during hypoxia [10]. Base excision repair (BER) pathway was shown to be functionally inactive during chronic hypoxia [11]. In prostate cancer cells, Meng *et al.* reported the downregulation of HR repair genes (e.g. *RAD51, RAD52, RAD54, BRCA1, BRCA2*) and NHEJ-repair genes (e.g. *XRCC6*, *PRKDC*, *LIG4*, *XRCC4*) following chronic hypoxia treatment of normal and malignant cells [12]. Curiously, it is not clear whether abrogation of double-strand

break repair would always promote cancer cell survival or cell death. For instance, loss of tumor suppressor genes such as *CDKN2A*, *TP53* occurs as cancer evolves and often lead to suppression of G1/S checkpoint activation [13]. Cancer cells lacking the G1/S checkpoint depend more on the G2/M checkpoint for cell survival as the G2/M checkpoint prevents mitotic entry of cells with DNA breaks, thereby protecting against mitotic catastrophe and cell death. If double-strand DNA repair pathways were completely inactive during hypoxia, cells enter mitosis with higher level of damaged DNA and are likely to die from mitotic catastrophe [14]. Therefore, perhaps low-level of double-strand break repair must be operational during hypoxia. Thus, there is further scope to investigate contribution (or the lack of it) of other DNA repair pathways in promoting genomic instability during hypoxia.

Non-heme dioxygenases: Non-heme Fe(II)/2OG-dependent dioxygenases constitute a large family of enzymes that catalyze broad range of oxidation reactions [15]. Structurally these proteins are characterized by eight antiparallel β-strands forming two β-sheets that commonly known as a jelly-roll motif. Sandwiched between the two β-sheets, an Fe(II) atom is bound via three conserved residues, a histidine, an aspartate/glutamate, and a histidine (HXD/E…H). These enzymes require Fe^{2+}, 2OG, O_2 and ascorbate. In addition to the primary substrate, 2OG is also a substrate for oxidation in the reaction; the 2OG is decarboxylated during the hydroxylation reaction to form CO_2 and succinate. One atom of the O_2 becomes integrated into the succinate and the other into the hydroxyl group generated on the substrate.

Functional inactivation of non-heme dioxygenases occurs during hypoxia: The enzymes belonging to non-heme Fe(II) and 2OG-dependent dioxygenases family, such as prolylhydroxylases (PHDs), are well known example of hypoxia regulated enzymes [16, 17]. Prolylhydroxylation is a post-translational modification that has been known for many years to occur in collagen proteins [18]. For collagen PHD, K_M (oxygen) was determined to be 40μM [19]. Ehrismann *et al.* measured K_M (oxygen) of variety of non-heme Fe(II)/??2OG-dependent dioxygenases and reported that, other than collagen PHDs, most enzymes belonging to non-heme dioxygenase family have K_M (oxygen) in the range of 70-90μM [20]. Oxygen concentration in air-saturated aqueous buffer at 37°C is approximately 200 μM (Figure 1A). However, it has been estimated that human tissue oxygen concentration is much lower and is in the range of 30-60μM. This corresponds to 7.5-15μM under moderate hypoxic condition. Therefore, all non-heme dioxygenase class of enzymes are most likely inactive during hypoxia (Figure 1A). PHDs also play a very important role in cellular adaptation to hypoxia. As mentioned before, cellular response to hypoxia is regulated by HIF-1α transcription factor. HIF-1α binds to HREs and upregulates broad range of transcriptional targets involved in cellular responses to hypoxia, such as angiogenesis and erythropoiesis [1] (Figure 1B). HIF-1α protein is constantly hydroxylated at a specific proline residue by the HIF-1α-specific PHD isoform-2 (PHD2) [21]. This modification increases the affinity of HIF-1α to the von Hippel-Lindau (VHL) E3 ubiquitin ligase, leading to its ubiquitination and proteasomal degradation [22]. K_M (oxygen) of HIF-1α-specific PHD2 was

determined to be in the range of 200-250μM [19, 20], which is well above the physiological concentration of oxygen (Figure 1A). This rather high K_M (oxygen) value suggests that, under physiological condition, activity of this enzyme is rate-limited by oxygen and perfectly designed for their role as oxygen sensors.

DNA demethylation, especially 5-methylcytosine (5mC) within CpG dinucleotides at gene promoters, is a dynamic epigenetic modification in mammalian DNA and is critical for transcriptional silencing of genes. DNA methyltransferases (DNMTs) catalyze DNA methylation, while Tet1–3 (Ten-eleven translocation) proteins catalyze conversion of 5mC to 5-hydroxymethyl-cytosine (5hmC) and further to 5-formyl-cytosine (5fC) and 5-carboxyl-cytosine (5caC) [23-27]. Once formed, 5fC and 5caC in DNA can be excised by a DNA glycosylase and subsequently repaired via BER. It has been shown that thymine-DNA glycosylase (TDG) possesses an excision activity towards 5fC- and 5caC-containing DNA [23, 28]. The Tet enzymes belong to non-heme Fe(II)/2OG-dependent dioxygenases family which need molecular oxygen for catalysis. If K_M (oxygen) for Tet enzymes is similar to other non-heme dioxygenase family members, then it is expected that they will be inactive under hypoxic condition. Indeed, it has been recently reported that Tet proteins become inactive under hypoxic condition (oxygen concentration 0.5%) and hypoxic tumors accumulate significant level of 5mC at gene promoters [29].

Like 5mC DNA methylation, dynamic methylation of mRNA at N6-methyladenosine (m6A) is frequently observed in mammalian cells. This m6A RNA modification is catalyzed by a methyltransferase enzyme complex

consisting of methyltransferase-like (METTL) proteins (METTL3 and METTL14) and Wilms tumor associated protein (WTAP) [30]. The m6A modification is reversed by m6A-specific non-heme Fe(II)/2OG-dependent dioxygenase family demethylases, AlkB homolog 5 (ALKBH5) and FTO, also known as AlkB homolog 9 (ALKBH9) [31-34]. It has been shown that hypoxia inactivates ALKBH5 enzyme and regulate the level of m6A modified mRNA [35]. This is yet another example of non-heme Fe(II)/2OG-dependent dioxygenases being inactive under hypoxic condition.

Besides prolylhydroxylation of collagen and HIF-1α, demethylation of 5mC at gene promoters and demethylation of m6A in mRNA, non-heme Fe(II)/2OG-dependent dioxygenases are also involved in direct DNA repair. *Escherichia coli* alkylation repair protein-B (AlkB), which is a Fe(II)/2OG-dependent dioxygenases family member, protects the bacterial genome against alkylation damage [36]. Exposure to alkylating agents from endogenous and exogenous sources results in carcinogenic mutations. Alkylated DNA adducts, induced by S_N2 alkylating agents, include 3-methylcytosine (3-meC) and 1-methyladenine (1-meA). AlkB catalyzes oxidative dealkylation in a reaction requiring oxygen, non-heme iron (Fe-II) (as cofactor), 2OG which serves as a co-substrate resulting in the formation of succinate and CO_2. When AlkB repairs 1-meA or 3-meC, the methyl group is first converted enzymatically to hydroxy-methyl group and being unstable in nature, this is rapidly removed as formaldehyde [37]; Most eukaryotes have AlkB homologs, including *Saccharomyces cerevisiae* [38]. Mammalian genome encodes three functional

AlkB homologs involved in DNA repair [39]. Among these, ALKBH1 displays mitochondrial localization and exhibit low-level of 3meC repair activity *in vitro* [40]. It was also shown that ALKBH1 has AP lyase activity [41] and is able to demethylate methylated histone H2A [42]. However, it is unclear whether ALKBH1 is a *bona fide* nuclear DNA repair enzyme. Only two AlkB homologs, namely ALKBH2 and ALKBH3, are the confirmed nuclear DNA repair enzymes [43-47]. Biochemical experiments indicate that ALKBH2 preferentially repairs double-stranded DNA (dsDNA) substrates, while ALKBH3 prefers single-stranded DNA (ssDNA) substrates [47] and RNA [43]. During S phase of cell cycle ALKBH2 interacts with the proliferating cell nuclear antigen (PCNA) via ALKBH2-PCNA-interacting motif (APIM) [48]. As PCNA slides along with replication fork, it is possible that interaction with PCNA may promote ALKBH2-mediated repair of dsDNA. ALKBH3 was found to be associated with the large subunit of activating signal cointegrator complex (ASCC3) [49]. It is likely that DNA helicase function of ASCC3 may generate ssDNA for ALKBH3 mediated repair.

Because ALKBH2 and ALKBH3 are structurally and functionally similar to PHDs, it is possible that under hypoxic condition these enzymes will also be completely or partially inactive like other non-heme Fe(II)/2OG-dependent dioxygenases. Moreover, these genes are not under HIF-1α control and remains unchanged during hypoxia [50]. The impact of inactivation of non-heme Fe(II)/2OG-dependent dioxygenases in promoting somatic mutagenesis and altering the physiology of the tumor during hypoxia remains an under-

investigated question.

Elevated level of expression of *ALKBH3* is reported in variety of cancers [51-54] and expression of *ALKBH3* is essential for survival of many cancer cells. Knockdown of *ALKBH3* resulted apoptosis in prostate cancer cell line DU145 [51] and non-small-cell lung cancer (NSCLC) cell line A549, which originated from a human lung adenocarcinoma. This cell line has wild type *TP53* and capable of p53-mediated apoptosis. Knockdown of *ALKBH3* in urothelial carcinoma cell line UM-UC3 resulted in cell cycle arrest and apoptosis [52]. Knock down of *ALKBH3* in a human renal cell carcinoma cell line, CAKI-1 inhibited cell proliferation [55]. Expression of *ALKBH3* was found to be essential for survival of colorectal cancer cell line DLD1 expressing mutant oncogenic *K-RAS,* but not for isogenic DLD1 cell line containing wild type *K-RAS* [56]. From these studies it can be proposed that some cancer cells are addicted to AlkBH3 function and this can be categorized as 'non-oncogene addiction'. AlkBH3 activity may not be essential for the viability of normal cells but is required to support the oncogenic phenotype of some cancer cells. Whether these cancer cells are addicted to ALKBH3 and particularly vulnerable to cell death due to functional inactivation of ALKBH3 activity during hypoxia remains to be examined.

Deficiency of DNA repair dioxygenases results in distinct mutation frequency: 3-meC and 1-meA lesions are predominantly generated in single-stranded DNA during replication, repair and transcription, and are efficiently repaired via direct oxidative reversal by non-heme Fe(II)/2OG-dependent

dioxygenases. One of the ubiquitous endogenous N-alkylating agents is S-adenosylmethionine (SAM) and it can nonenzymatically methylate DNA [57]. Interestingly, all three catalytically active DNMTs, *DNMT1*, *DNMT3A*, and *DNMT3B*, use SAM as methyl donor. Presence of functional DNMTs during DNA replication implicates the presence of SAM in the vicinity; therefore, SAM could nonenzymatically methylate DNA during replication. In addition, *in vivo* lipid peroxidation reactions and nitrosation of amines could give rise to reactive methylating species. Not surprisingly, spontaneously arising 1meA lesions were shown to accumulate in the genome of the *Alkbh2* knockout mice [58]. Formation of 1-meA and 3-meC at DNA base-pairing positions prevents Watson-Crick base pairing, thereby stalls DNA replication and results in recruitment of TLS DNA polymerases [59]. Knockout mice with deletion of *Alkbh2* and *Alkbh3* results in spontaneous accumulation of higher level of C→A transversion and C→T transition mutations [46]. *In vitro* studies by Furrer *et al.* have revealed the role of Y-family trans-lesion synthesis (TLS) polymerases in development of mutagenesis and shown that DNA polι preferentially misincorporates dTTP and Polη dATP opposite to 3-meC [59], explaining the accumulation of C→A and C→T mutation in *Alkbh2*$^{-/-}$ and *Alkbh3*$^{-/-}$ mice. This mutational pattern coexists with other mutations in many cancer types and could be partially contributed by the hypoxic inhibition of ALKBH2 and ALKBH3. Because we do not know the K_M (oxygen) of ALKBH2 or ALKBH3, the extent of inhibition cannot be known accurately. However, given the structural similarity of ALKBH2 and ALKBH3 with PHDs, we may assume that hypoxia would result in significant functional

impairment of ALKBH2 and ALKBH3 activity (Figure 1B). It is not clearly known whether other human AlkB homologues are also inactivated under hypoxic condition. *In vitro* experiments have shown that human AlkB homolog ALKBH5 becomes inactive under hypoxic condition (1% oxygen concentration) [35]. However, hypoxia is also known to stimulate ALKBH5 expression which lead to the demethylation of m6A modification of Nanog mRNA [34].

Large-scale genome-wide sequence analyses in last few years have revealed 30 mutational signatures across the spectrum of human cancer types. One such mutation pattern is now referred to as “signature 10” in the COSMIC (catalogue of somatic mutation in cancer) database. This distinct signature mutations, found in six different types of cancer but common in colorectal and endometrial cancers, is characterized mainly by a prevalence of C→A transversion and C→T transition mutations [60]. Cancer genome sequencing studies as part of the TCGA (The Cancer Genome Atlas) have revealed that C→A and C→T mutations in colorectal and endometrial cancers are associated with the mutations in the proofreading domain of DNA polymerase-epsilon (polε) [61-66]. Mutation in polε is associated with high mutagenic potential resulting significant level of genomic instability [67, 68]. Because the mutation pattern in *Alkbh2* and *Alkbh3* knockout mice is similar to the mutation pattern found in polε mutant endometrial and colorectal cancers, one would wonder what happens when these causes are present simultaneously. It may be speculated that rather strong mutator phenotype will emerge if polε mutation was combined with ALKBH2 and ALKBH3 inhibition during hypoxia. Curiously, hypoxia is frequently

observed during development of endometrial and colorectal cancer [69, 70]. It remains to be experimentally proven whether inactivation of ALKBH2 and ALKBH3 during hypoxic phase renders polε mutant endometrial and colorectal cancer cells accumulating extraordinarily high number of cancer-driving mutations.

Conclusion: Activity of human DNA repair proteins ALKBH2 and ALKBH3, which belongs to non-heme dioxygenase family, depends on molecular oxygen. During hypoxia, loss of ALKBH2 and ALKBH3 activity may result alkylation adducts on DNA. This may result in error-prone bypass replication and accumulation of mutations. Thus, the loss of ALKBH2 and ALKBH3 activity during hypoxia may create a mutator phenotype and cause large number of somatic mutations found in hypoxic cells. It may also be one of the factors responsible for manifestation of hypoxia-associated malignant phenotype and aggressive tumor behaviour.

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Conflict of interest:

None.

Reference:

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Figure legends

Figure 1. Regulation of oxygen-dependent non-heme dioxygenases.

(A) Outline of the cellular oxygen concentration and K_M (oxygen) of different classes of Fe(II)/2OG-dependent dioxygenases. K_M (oxygen) values were obtained from reference [15] and [16]. **(B)** Schematic representation of causes and consequences of inhibition of Fe(II)/2OG-dependent dioxygenases, such as ALKBH2 and ALKBH3.

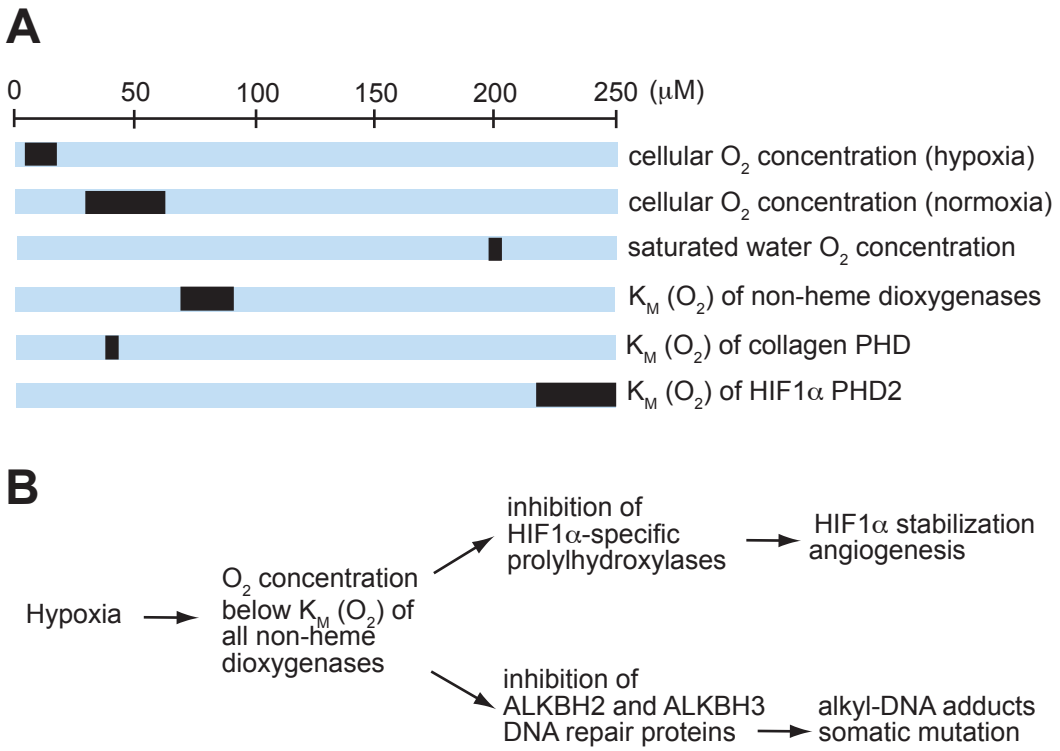

Figure 1