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**EDITORIAL**

# TUMOR HYPOXIA IS IMPORTANT IN RADIOTHERAPY, BUT HOW SHOULD WE MEASURE IT?

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## INTRODUCTION

The issues surrounding tumor oxygenation and radiotherapy used to be simple. Hypoxic cells are radioresistant and are present in a large proportion of human solid tumors; in addition, overall tumor oxygenation (as determined by a commercially available oxygen electrode, commonly referred to as the Eppendorf electrode) correlates with response to radiotherapy. Because of its correlations with clinical outcomes (particularly in head-and-neck tumors), the Eppendorf electrode has become "the gold standard" against which all other techniques are to be compared. Another appealing feature of the Eppendorf electrode is its ability to provide a quantitative distribution of real oxygen levels in tumors.

However, it is not without problems. First, it can be applied only to superficially accessible tumors. Second, it requires considerable operator experience for proper use (something that is rarely discussed in the literature), and in many instances, image guidance is required for proper placement of the electrode. Most problematic, however, is that it cannot distinguish between tumor tissue and necrosis, which can bias the relevant oxygenation values to spuriously low levels in tumors with extensive necrosis.

Because of these problems with the Eppendorf electrode, there are a number of ongoing efforts to find alternative methods of estimating tumor oxygenation. Two of the main candidates that have been used clinically over the past 2 to 3 years are nitroimidazole hypoxia markers and the so-called "endogenous markers" of tumor oxygenation. The first of these requires injection of a compound (a nitroimidazole such as pimonidazole or EF5) some hours before tumor resection or biopsy for immunohistochemical detection of the bound nitroimidazole derivative that occurs only in hypoxic tissues.

The second of the alternatives to the Eppendorf is the use of endogenous markers, or proteins induced by hypoxia, such as the transcription factor HIF-1$\alpha$ or genes transcribed by HIF-1, such as carbonic anhydrase 9 (CA9). The levels of both of these proteins are increased under hypoxic conditions, and both can be detected in tumor sections by immunohistochemistry. The huge advantage of this latter approach is that levels of these proteins can be assessed on archival materials, thereby allowing rapid correlation to treatment outcomes. In addition, this approach requires neither the injection of foreign material nor any additional invasive procedure beyond that of taking a tumor biopsy at diagnosis.

The overriding goal of all of these studies is a simple, widely usable, and robust method of assessing tumor oxygenation that will correlate with clinical outcomes. But here is where it gets more complicated. First, there are at least two distinct forms of tumor hypoxia. The first of these, described by Thomlinson and Gray 50 years ago, is one in which hypoxic cells were postulated to occur beyond the diffusion distance of oxygen from blood vessels and adjacent to necrotic areas. This is now known as chronic hypoxia. The second is acute hypoxia. It has been shown both in experimental animals and in human tumors that tumor blood flow varies over time, and (at least in animal tumors) this gives rise to hypoxia caused by the temporary reduction in flow or closure of certain blood vessels within the tumor. It must be borne in mind, however, that acute and chronic hypoxia are likely to be the extremes of a continuum caused by the dynamic nature of tumor blood flow, which causes variations in the diffusion of oxygen in at least some of the tumor vessels. The reason why having two forms of tumor hypoxia is a complicating factor is because we do not know whether both of these forms of hypoxia are equally important for radiotherapy outcome or whether one is more important than the other.

The second reason for the increasing complexity of the field is that the different methods of assessing tumor oxygenation measure acute and chronic hypoxia to different extents. In the case of the Eppendorf electrode, the mea-

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surements reflect both acute and chronic hypoxia (as well as the irrelevant necrotic areas, which are counted as hypoxic, as was mentioned earlier). The hypoxia markers pimonidazole and EF5 measure primarily chronic hypoxia. The endogenous markers HIF-1$\alpha$ and CA9 would also be expected to measure chronic hypoxia, although many other genetic alterations in the tumor cells can affect the expression of these proteins. Of note is that there is at present no clinically usable way of estimating the proportion of acute hypoxia in human tumors.

The paper by Janssen *et al.* in this issue (Reference) describes two novel approaches that attempt to measure acute and chronic hypoxia separately in human head-and-neck tumors. The first new method uses an image analysis estimation of the diffusion-limited fraction (DLF), defined as the proportion of total tumor tissue greater than 120 $\mu$m from the nearest vessel, to estimate the fraction of chronically hypoxic tumor cells. The second method, which is used to measure acute hypoxia, is based on a previous finding by the authors that staining for the proliferation marker IdUrd after i.v. injection of this DNA base analog is heterogeneous in the tumor, suggesting that some blood vessels were closed at the time of injection and, therefore, the IdUrd was not available to the cells surrounding those vessels. The authors showed by staining with the endogenous proliferation marker Ki-67 that in almost all cases, this lack of IdUrd staining around individual blood vessels was not because of the lack of tumor cell proliferation, and hence, it was likely because of a lack of marker availability as a result of deficient blood flow in that vessel. In addition to these novel methods, the authors stained the sections for pimonidazole binding (injected along with the IdUrd the evening before surgery) and for the endogenous marker HIF-1$\alpha$, two commonly used methods of estimating chronic hypoxia.

How well did the different ways of estimating chronic hypoxia agree with each other? First, there was no correlation of the mean values of DLF and pimonidazole binding between the 14 evaluable tumors, though there was a correlation between individual values of DLF and pimonidazole binding in individual areas in 7 of the 14 tumors. It is unclear at the moment which of the two is the better marker for chronic hypoxia, because both have potential problems: DLF could be compromised by out-of-section blood vessels, and pimonidazole could be potentially compromised by staining of areas of keratinization, which may or may not be hypoxic. However, the authors' finding that pimonidazole binding rises sharply only after 80 $\mu$m from blood vessels suggests that this marker might be the more reliable of the two. Second, there was no correlation between the levels of pimonidazole binding and HIF-1$\alpha$ staining for the tumors. Further, in sharp contrast with the distribution of pimonidazole binding in perinecrotic areas (as expected), HIF-1$\alpha$ staining only peaked distally from blood vessels in 1 of the 6 evaluable tumors. In the others, the distribution of this protein was largely uniform as a function of distance from the vasculature. Based on these data, HIF-1$\alpha$ would not therefore seem to be a reliable estimator of chronic hypoxia.

What of acute hypoxia? Janssen *et al.* found a wide variation (0.7%–32.5%) in the number of blood vessels not surrounded by IdUrd-labeled cells and, therefore, assumed to be not perfused at the time of labeling. This would represent an estimate of the proportions of acute hypoxic cells in these tumors, but there are no other measurements to corroborate these findings. It will be of great interest to see from future studies by these investigators the extent to which this estimator of acute hypoxia correlates with treatment outcome.

How do these data fit with others in the literature? The ability to perform retrospective studies of outcomes with HIF-1$\alpha$ and CA9 immunostaining of archival tumor material has produced a rash of recent papers correlating these endogenous markers with outcomes of treatments in a variety of tumors. In general, we can conclude from these studies that HIF-1$\alpha$ staining is inferior to CA9 as a marker of chronic hypoxia, because of its homogeneous staining pattern in relation to the vasculature when compared to CA9, which generally is perinecrotic in distribution and colocalizes better with pimonidazole. These results have been reported for both cervix and head-and-neck cancers. This may be because of an additional oxygen-sensitive step for transactivation of HIF-1$\alpha$ target genes (1), which results in better CA9 colocalization with hypoxia. However, before we dismiss HIF-1$\alpha$ as a hypoxia marker, in a much larger series of head-and-neck cancers than that of Janssen *et al.*, Aebersold *et al.* (2) observed both perinecrotic (in 2/3 of the tumors) and diffuse (in 1/3 of the tumors) staining patterns for HIF-1$\alpha$, so it is possible that with only 6 evaluable patients, Janssen *et al.* by chance may have overestimated the proportion of tumors with diffuse HIF-1$\alpha$ staining. Also, Aebersold *et al.* found that HIF-1$\alpha$ staining was predictive for outcome in oropharyngeal cancers treated with radiotherapy (2). This is probably a reflection of the radiation resistance of hypoxic cells, because HIF-1$\alpha$ overexpression has been correlated with *improved* survival for head-and-neck cancer patients treated with surgery alone (3). Though this would seem to contradict the dogma that hypoxic tumors are intrinsically more aggressive, it is consistent with the report of Aebersold *et al.*, who found a reverse correlation between HIF-1$\alpha$ and grade (2).

In summary, we can conclude that HIF-1$\alpha$ staining has some correlation with tumor oxygenation (also shown by Haugland *et al.* for cervix cancer [4]), but it is clearly inferior to CA9 as a pure hypoxic marker in human solid tumors. However, the jury is still out as to which of all the hypoxia markers will be a better predictor of outcome for radiotherapy or for surgery. Certainly, the flood of new data with the endogenous hypoxia markers, as well as the new approaches described by Janssen *et al.*, are exciting and promise to lead to a simple method of assessing tumor oxygenation that can be used on all patients. Such a marker will not only aid in prognosis, it will also be a big help in deciding which patients should have a hypoxia-directed treatment as part of their overall management.

## REFERENCES


1. Lando D, Peet DJ, Whelan DA, *et al.* Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 2002;295:858–861.
2. Aebersold DM, Burri P, Beer KT, *et al.* Expression of hypoxia-inducible factor-1alpha: A novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. *Cancer Res* 2001;61:2911–2916.
3. Beasley NJ, Leek R, Alam M, *et al.* Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: Relationship to tumor biology and treatment outcome in surgically resected patients. *Cancer Res* 2002;62:2493–2497.
4. Haugland HK, Vukovic V, Pintilie M, *et al.* Expression of hypoxia-inducible factor-1alpha in cervical carcinomas: Correlation with tumor oxygenation. *Int J Radiat Oncol Biol Phys* 2002;53:854–861.