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PUSHING THE FRONTIERS OF RADIOBIOLOGY: A SPECIAL FEATURE IN MEMORY OF SIR OLIVER SCOTT AND PROFESSOR JACK FOWLER: REVIEW ARTICLE

Hypoxia gene expression signatures as predictive biomarkers for personalising radiotherapy

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ABSTRACT

Hypoxia is a generic micro-environmental factor of solid tumours. High levels of hypoxia lead to resistance to radiotherapy, which can be targeted by adding hypoxia-modifying therapy to improve clinical outcomes. Not all patients benefit from hypoxia-modifying therapy, and there is a need for biomarkers to enable progression to biologically personalised radiotherapy. Gene expression signatures are a relatively new category of biomarkers that can reflect tumour hypoxia. This article reviews the published hypoxia gene signatures, summarising their development and validation. The challenges of gene signature derivation and development, and advantages and disadvantages in comparison with other hypoxia biomarkers are also discussed. Current evidence supports investment in gene signatures as a promising hypoxia biomarker approach for clinical utility.

INTRODUCTION

In the field of radiation oncology, there is a strong rationale behind investing research effort into measuring tumour hypoxia. First, there is a high level of evidence showing that hypoxia modification of radiotherapy improves both local control and overall survival. Second, an increasing body of data shows that patients with the most hypoxic tumours benefit the most from hypoxia-modifying treatments. Third, faced with an increasing number of agents to combine with radiotherapy, there is a need to progress towards personalised medicine and biomarker-driven trials. This review provides an overview of the development of gene expression signatures for assessing tumour hypoxia, which are argued to have the greatest potential for clinical application as biomarkers to personalize radiotherapy.

TUMOUR HYPOXIA AND RADIOTHERAPY RESPONSE

Interest in hypoxia spans six decades since implications for its role in radiotherapy resistance were made following observations of the histological structure of human lung cancers.¹ For many years it has been known that oxygen depletion reduces the formation of DNA strand breaks by sparsely ionising radiation² and the effectiveness of radiotherapy. The last 20

years also saw increasing recognition in the wider oncology field for the importance of hypoxia, which is now accepted as a hallmark of solid cancers.³ Sustained hypoxia leads to a more aggressive tumour phenotype and higher potential for metastatic spread.^{4,5}

Demonstration of the radioresistance of hypoxic cells underpinned the development of hypoxia-targeting therapies.⁶ The approaches studied include increasing oxygen delivery, sensitising hypoxic cells to radiation or using bioreductive agents that are selectively cytotoxic to hypoxic tumour cells.^{5,7–9} Phase III trials provide a high level evidence for the benefit of combining radiotherapy with agents that modify hypoxia.^{5,10} A meta-analysis was carried out of 86 Phase III trials comparing radiotherapy with curative intent alone or with hypoxia modification.¹⁰ Hypoxia modification improved both loco-regional control [odds ratio (OR) 0.77, 95% CI 0.71–0.83] and overall survival [OR 0.87, 95% CI 0.80–0.95]. Similarly, Salem et al⁵ observed comparable effect sizes of hypoxia modifiers for both endpoints in randomised trials of non-small-cell lung cancer patients. Despite ample evidence, hypoxia-targeting therapy has not yet progressed into widespread clinical practice.

BIOMARKERS

A biomarker is an objective indication of medical state that can be measured with accuracy and reproducibility¹¹ and used to identify a disease or indicate treatment response.¹² For patients diagnosed with malignancy, clinical biomarkers fall into two broad categories—prognostic and predictive markers.¹³ A prognostic biomarker provides the likelihood of patient outcome regardless of the therapy, *e.g.* a biomarker that can predict the probability of recurrence for patients receiving either radiotherapy alone or radiotherapy with hypoxia modification is only prognostic. A predictive biomarker, however, provides the likelihood of patient response to a particular therapy, *e.g.* a biomarker is predictive if adding hypoxia modification reduces the recurrence rate in biomarker-positive patients but not in biomarker-negative patients. Predictive biomarkers are more useful clinically but much more challenging to develop and validate.

In cancer research, the number of biomarker-related publications surged from about 10 in 1990 to over 4000 in 2016 (PubMed search: “biomarker” AND “cancer” or “carcinoma” in Title or Abstract). Despite the extensive interest, very few biomarkers are used in routine clinical practice. The number of clinically validated predictive biomarkers is even lower. The lack of biomarker translation into the clinic highlights the need for more efficient strategies for their development and evaluation.

HYPOXIA BIOMARKERS

The first reports measuring oxygen tension in human tumours are credited to Urbach who studied skin malignancies¹⁴ and Kolstad¹⁵ who showed an effect of hypoxia on radiocurability in cervix cancer. However, it is the work led by Peter Vaupel and Michel Hockel¹⁶ using computerised fine needle electrodes that led to a resurgence of interest in measuring tumour hypoxia at the turn of the century. Subsequently, numerous approaches have been developed and studied for assessing tumour hypoxia, including needle electrodes,^{17–19} pimonidazole,^{18,20,21} imaging,^{22–24} protein markers^{25–29} and more recently gene expression signatures.^{20,30–33}

Hypoxia biomarkers have already shown clinical relevance. Direct measurements of tissue oxygenation with polarographic electrodes showed that the level of hypoxia varies considerably across tumours.^{18,19,34} In multiple cancers, hypoxia biomarkers were associated with independent prognostic value.^{4,18,30,35–37} Studies also indicated that severe resistance to radiation was limited to pO₂ levels < 0.13%.⁷ Several studies suggested that only the most hypoxic tumours benefited from hypoxia-targeting therapies^{25,30,31,33,38,39} (Table 1).

It is however disappointing that, despite years of research, there is still no reliable way of patient stratification for hypoxia-targeting therapy.²² The absence of success could perhaps be attributed to the fact that most hypoxia biomarker research focuses on development rather than clinical validation, either due to lack of access to clinical cohorts or due to technical difficulty. There is increasing evidence that hypoxia gene expression signatures have potential for application as clinical biomarkers.

Table 1. Studies showing that only the most hypoxic tumours benefit from hypoxia-targeting therapy

	Cancer	Hypoxia modifier	Endpoint	Biomarker	Effect in Biomarker-positive patients	Effect in Biomarker-negative patients
Rischin ³⁸	Head and neck	Tirapazamine and cisplatin or cisplatin and infusional fluorouracil	Loco-regional relapse	FMISO-PET	N = 32, HR 0.067 (<i>p</i> = 0.001)	N = 13, no conclusion derived
Janssens ³⁹	Head and neck	Carbogen and nicotinamide	Regional relapse	Pimonidazole	N = 26, 100% vs 55% log rank <i>p</i> = 0.01	N = 53, 96% vs 92% log rank <i>p</i> = 0.7
Hunter ²⁵	Urothelial	Carbogen and nicotinamide	Local relapse	HIF-1 α protein	N = 68, HR 0.48 (0.26–0.8, <i>p</i> = 0.02)	N = 69, HR 0.81 (0.43–1.50, <i>p</i> = 0.5)
Eustace ³³	Head and neck	Carbogen and nicotinamide	Regional relapse	26-gene signature	N = 78, HR 0.14 (0.03–0.62, <i>p</i> = 0.009)	N = 79, HR 1.10 (0.25–4.76, <i>p</i> = 0.90)
Toustrup ³⁰	Head and neck	Nimorazole	Loco-regional relapse	15-gene signature	N = 114, HR 0.42 (0.25–0.68)	N = 209, HR 0.98 (0.67–1.44)
Yang ³¹	Urothelial	Carbogen and nicotinamide	Local relapse	24-gene signature	N = 76, HR 0.47 (0.26–0.86, <i>p</i> = 0.015)	N = 75, HR 1.52 (0.79–2.93, <i>p</i> = 0.21)

Table 2. Summary of published hypoxia gene signatures derived using clinical samples

	Cancer	Size	Outcome information used in gene selection	Expression summarisation	Hypoxia status	Prognostic	Predictive
Winter ³²	Head and neck	99	N	Median	Continuous	Y	N
Toustrup ³⁰	Head and neck	15	N	Formula	Binary	Y	Y
Eustace ³³	Head and neck and breast	26	N	Median	Continuous	Y	Y
Suh ⁴⁴	Head and neck	21	N	Mean	Continuous	Y	N
Halle ⁴⁵	Cervix	31	Y	Mean	Continuous	Y	N
Fjeldbo ⁴⁶	Cervix	6	Y	Formula	Binary	Y	N
Ragnum ²⁰	Prostate	32	N	Median	Continuous	Y	N
Yang ³¹	Urothelial	24	Y	Median	Continuous	Y	Y
Yang ⁴⁷	Soft tissue sarcoma	24	N	Formula	Binary	Y	N

HYPOXIA GENE EXPRESSION SIGNATURES

Cancer cells alter the transcriptomic network when adapting to hypoxia. Several biological processes are activated under hypoxia, including HIF-1 pathways, glycolysis, apoptosis and angiogenesis.^{40,41} Hypoxia induces the expression (mRNA abundance) of a large number of genes, including *CA9*, *GLUT1*, *VEGF*, *ADM* and *AK3*.^{32,42} The emergence of hypoxia signatures is largely due to the development of high-throughput expression profiling technologies. Either micro-array or RNA sequencing can reliably measure the transcriptome (>20,000 protein-coding genes) of a tumour sample. A gene expression signature refers to multiple genes whose expression collectively indicates a particular phenotype of interest.⁴³

The first step in deriving a hypoxia gene signature involves identifying a set of genes most likely to be correlated with hypoxia in patient tumours. The expression levels of those signature genes from an individual tumour are then summarised into a single score.

HOW HYPOXIA GENE EXPRESSION SIGNATURES ARE GENERATED

A recent review listed 32 hypoxia gene expression signatures published between 2000 and 2013,⁴² many of which were derived solely from cell lines. Here, we focus only on the work where patient cohorts were analysed in both the signature derivation and validation processes, regardless of whether *in vitro* data were generated for signature derivation. Nine signatures, summarised in Table 2, were identified for cancers of the head and neck ($n = 4$), cervix ($n = 2$), prostate ($n = 1$), bladder ($n = 1$) or soft tissue ($n = 1$).

Our group and collaborators derived the first hypoxia gene signature for patients with head and neck squamous cell carcinoma in 2007.³² 10 well-known hypoxia inducible genes, including *CA9*, *ADM*, *VEGF*, *GLUT1*, were curated as seed genes and a 99-gene signature was identified whose mRNA expressions correlated well with the 10 seed genes in patient samples. The signature had prognostic value in an independent validation cohort.

The process was repeated in multiple cohorts of breast and head and neck cancers to derive both common and cancer-specific

gene signatures. Genes in the signatures were then ranked according to their co-expression with hypoxia seed genes (genes co-expressed with more seed genes were deemed to have higher connectivity).⁴⁸ Prognostic significance was tested in multiple independent cohorts. Cumulative forest plots were generated with genes added one by one in the order of decreasing connectivity. Prognostic significance stabilised after a few genes were added and reduced signatures were equally prognostic to the full signatures. Our group took forward the head and neck signature for further development using the top 26 genes from the gene rankings.³³ The decision to use 26 genes was based on the 384-sample array selected for clinical application. Customised arrays were made that allowed four samples to be assayed in triplicate with 26 signature genes, five internal reference genes and a plate control. Samples from 157 T_2 – T_4 laryngeal cancer patients enrolled in a randomised trial were used to validate the 26-gene signature. In patients with high 26-gene signature scores (split using the cohort median), 5-year regional control improved from 81 to 100% ($p = 0.009$) for patients having radiotherapy alone or with carbogen and nicotinamide, respectively. In patients with low 26-gene signature scores, no benefit from hypoxia modification was observed with regional control rates of 91% (radiotherapy alone) versus 90% (radiotherapy plus carbogen and nicotinamide). Therefore, the signature predicted the benefit for giving hypoxia-targeting therapy with radiation, although a formal interaction test could not be performed owing to low event rates. Importantly, the validation was performed in pretreatment formalin-fixed paraffin-embedded (FFPE) blocks, the routine procedure for storing tissue samples in hospital. Also, the work was carried out under Good Clinical Practice laboratory standards to enable subsequent prospective clinical qualification.

Overgaard's group developed an alternative hypoxia signature for head and neck cancer.³⁰ Candidate genes inducible by hypoxia and independent from pH regulation were first identified from *in vitro* experiments.⁴⁹ In a subsequent study, paired gene expression and ¹⁸F-fluoroazomycin arabinoside imaging biomarker data were obtained from xenograft models. Candidate genes were all confirmed to be upregulated under hypoxia *in vivo*. Finally, in a cohort of tumour biopsies with paired pO_2 measurement and gene expression data, the candidate genes

were refined into a 15-gene signature that maximally separated the most hypoxic tumours from the rest. The signature was validated in a cohort of 323 patients enrolled in a randomised trial of radiotherapy with or without nimorazole. In patients with high 15-gene signature scores, 5-year loco-regional failure decreased from 79 to 46% with the addition of nimorazole. In patients with low 15-gene signature scores, nimorazole did not lead to a significant decrease in loco-regional failure (46%) compared with radiotherapy alone (54%). A formal interaction test was significant with $p = 0.003$.

Suh et al⁴⁴ performed paired hypoxia imaging using ⁶⁴Cu-ATSM PET/CT and whole transcriptome gene expression profiling in 15 patients. 21 genes correlated or anticorrelated with the imaging biomarker and formed a signature, which was prognostic for 3-year progression-free survival in a cohort of patients treated with surgery.

Lyng's group performed dynamic contrast-enhanced MRI (DCE-MRI) in 78 locally advanced cervix cancer patients and generated whole transcriptome expression data in a sub-cohort.⁴⁵ Gadopentetate dimeglumine was used as contrast agent and a series of images acquired over time to generate time-concentration curves, which can be analysed (there are several models) to obtain vascular parameters that reflect, for example, blood perfusion and the volume of the extracellular space. A revised DCE-MRI parameter, calculated as the mean value over 20–30% of the tumour histogram of A_{Brix} , was found to have the optimal prognostic value. Genes were then ranked by their association with the revised A_{Brix} parameter, where strongly associated genes were enriched in hypoxia-regulated gene sets, including cervical-cancer-specific hypoxia-regulated genes established from cell lines. A 31-gene signature was derived from four hypoxia-related gene sets associated with the prognostic imaging parameter of revised A_{Brix} . The prognostic value of the signature was validated in an independent chemoradiotherapy-treated patient cohort. In a later paper, the group refined the 31-gene signature into a 6-gene signature, the prognostic significance of which was further validated.⁴⁶

The Lyng laboratory subsequently identified a gene signature that reflected pimonidazole staining in patients with localised prostate cancer.²⁰ Pimonidazole staining and transcriptome expression profiling data were obtained for 39 primary prostate cancers. Genes correlating with higher pimonidazole scores and representing proliferation, DNA damage repair and hypoxia pathways formed a 32-gene signature. In an independent cohort, the 32-gene pimonidazole signature was prognostic for patient overall survival.

Our group recently identified a hypoxia signature in bladder cancer.³¹ Over 250 genes known to be regulated by hypoxia from the literature were used as candidates. Candidate hypoxia genes that strongly correlated with each other and associated with a poor prognosis were taken as signature genes. The 24-gene signature was prognostic for survival in four independent cohorts of patients treated with radical cystectomy. Predictive capacity of the signature was further validated in 151 patients enrolled in a

randomised trial of radiotherapy with or without carbogen and nicotinamide.⁵⁰ In patients with high 24-gene signature scores, hypoxia modification improved local recurrence-free survival with a hazard ratio of 0.47 ($p = 0.015$). In patients with the low 24-gene signature scores, no significant benefit was observed ($p = 0.21$). A formal interaction test was also significant ($p = 0.0094$).

We also derived a 24-gene hypoxia signature for soft tissue sarcoma tumours.⁴⁷ First, hypoxia inducible genes were identified from seven soft tissue sarcoma cell lines exposed to hypoxia. 33 protein-coding genes consistently upregulated in all cell lines were curated as seed genes. In a patient cohort, tumours were clustered into two phenotypes based on the expression similarity of the 33 seed genes. Most of the seed genes were significantly upregulated in one tumour cluster, which was also enriched with a larger number of published hypoxia gene sets. A 24-gene signature was derived that best predicted the cluster membership of tumours in the training cohort. The signature was independently prognostic of distant-metastasis-free survival in the training and two independent validation cohorts.

As discussed above, the methodologies employed for hypoxia signature derivations varied considerably and are summarised in Figure 1. The differences were largely due to (1) the different underlying rationale, (2) the different wet laboratory data available, (3) the inherent diversity of gene expression signatures and (4) whether patient outcome information was used during the signature derivation. First, the nine *in vivo* derived signatures could be divided into two categories. Four signatures were developed by analysing the expression similarity of hypoxia regulated genes,^{31–33,47} since tumours highly expressing multiple hypoxia-inducible genes are more likely to be hypoxic. The other five signatures were derived by identifying genes whose expression correlated with other hypoxia biomarkers (pO_2 , pimonidazole or imaging markers),^{20,30,44–46} therefore, they serve as surrogates of other biomarkers less practical for widespread clinical use.

Available wet laboratory data also affects the signature development. For the group of four signatures derived without referencing to external biomarkers, their methodological differences mainly lie on whether seed genes are curated from the literature or from *in vitro* identification of hypoxia-regulated genes. In Toustrup et al,³⁰ *in vitro* hypoxia genes were first identified using cell lines exposed to hypoxia with *in vivo* upregulation confirmed in xenograft models. The signature was derived by correlating candidate genes with tumour biopsy pO_2 measurement. In three other papers,^{20,45,46} *in vitro* hypoxia genes were identified and used for filtering the genes correlated with either a prognostically selected imaging parameter or pimonidazole. In Suh et al⁴⁴ no *in vitro* data were used.

Third, different methods were used to summarise expression levels of signature genes into one signature score per tumour, including mean, median or using more complicated mathematical formula. While a median value is more robust than mean values to outliers, additional expression scaling (for example popularly used gene-wise centring) would undesirably alter the relative rank of the signature scores. We used the term

Figure 1. Methodologies employed to generate different hypoxia gene signatures. DCE-MRI, dynamic contrast-enhanced MRI; PET/CT, positron emission tomography/CT.

“formula” in a loose manner that includes all signatures where genes expression were summarised into signature scores using an explicit mathematical formula. Formula-based signatures were derived in three studies.^{30,46,47} Although not a necessity, formula-based signatures were frequently designed to produce binary outcome, i.e. high-hypoxia and low-hypoxia. Calculation of median or mean values yields continuous signature scores, which could be binarised from a prespecified cut-off (e.g. cohort median). Both approaches are effective when used for signature validation in a retrospective cohort. It is worth noting that for prospective evaluation in both biomarker-driven randomised trials and retrospective evaluation in prospective randomised trials, the predefined cut-off values used from the continuous or binary signature scores would affect the sample size and power calculation.¹³

Last, the nine signatures could also be stratified into two groups based on whether patient outcome information was used for signature gene selection. In the development of three signatures,^{31,45,46} putative hypoxia genes were selected by their prognostic value, either directly or indirectly via correlating with imaging biomarkers identified based on prognostic value. The underlying rationale is that hypoxia leads to an aggressive phenotype and resistance to radiotherapy, and thus is likely to correspond to a poor prognosis phenotype clinically. The other six signatures were derived without taking into account the patient outcome data following radical therapy and were entirely based on analysing gene co-expression structure or correlation with other hypoxia biomarkers.

CHALLENGES OF DEVELOPING AND VALIDATING HYPOXIA GENE SIGNATURES

One problem associated with all gene-expression-based studies is that the current expression profiling platforms all measure relative rather than absolute mRNA abundance.⁵¹ Measured mRNA

abundance depends on the preservation technique (fresh frozen or FFPE), age of the FFPE blocks, technical batch effect etc. This essentially means that, for the same gene signature, signature scores of different cohorts (e.g. training and different validation cohorts) are theoretically incomparable.

Another problem is the transferability across different tumour sites. We previously showed that most of the hypoxia signatures derived for other cancers did not predict the benefit of hypoxia-targeting therapy in bladder cancer.³¹ Other studies also appeared to indicate that transcriptomic response to hypoxia varies across different cancers,^{42,52} although more research is required to yield a comprehensive picture of gene expression association with hypoxia in a pan-cancer setting.

There is also the difficulty of benchmarking hypoxia signatures against the generally considered gold standard criterion, i.e. needle electrode measurements of pO₂. Measurement of tumour oxygen partial pressure using needle electrode is technically demanding especially for large-scale validation studies. Therefore, it is difficult to evaluate associations between hypoxia assessed using markers such as gene expression signatures and gold standard oxygen electrodes. The study of Toustrup et al.³⁰ remains the only study that derived a gene expression signature that reflected pO₂ measurements in human tumours.

Furthermore, whether patient outcome data should be used for signature gene selection could also be debated. Gene selection based on prognostic information^{31,45,46} has the advantage of deriving a clinically relevant prognostic biomarker, while one might also question their hypoxia specificity. Do they reflect prognostic parameters other than hypoxia? Or do they reflect the most clinically important hypoxia, which is sufficient to generate differential patient survival? More systematic research is required to fully understand those questions.

Last, clinical validation of hypoxia biomarkers is challenging. Evaluation of prognostic significance is relatively straightforward as it only requires observational cohorts. Nevertheless, most publicly available gene expression datasets that include outcome information are surgical cohorts. There is a need to generate more cohorts for radiotherapy patients with well-annotated outcome data. There is also the issue that prognostic significance does not guarantee predictive significance. Any predictive biomarker requires evaluation in a cohort of patients treated with different interventions. Therefore, a hypoxia biomarker needs to be evaluated in patients treated with or without hypoxia-targeting therapies in order to assess any predictive value. The validation cohort is preferably from a randomised Phase III trial of radiotherapy alone and radiotherapy plus hypoxia modification or might be a well-matched cohort.¹³ A hypoxia biomarker is predictive if the effect of hypoxia modification is statistically different in patients of different biomarker status (binary or continuous). Both are difficult to acquire, making it a difficult task to conclude definitively the clinical value of a hypoxia biomarker. The only cohort available from a randomised trial with full transcriptomic data is the BCON bladder cancer cohort.³¹

PROSPECTIVE APPLICATION OF HYPOXIA SIGNATURES

Prospective clinical use of a gene signature also requires assay development. In Toustrup,³⁰ a gene expression assay was developed and used in both signature training and validation. Therefore, the assay could be directly used for prospective patient stratification.

For the other signatures where the final application platform differs from the platform of training samples, a cohort (50–100) of tumour FFPE blocks should be collected and have their signature gene expressions generated using the final application assay. In Fjeldbo⁴⁶ the 6-gene signature was developed first from Illumina whole transcriptome platform, where in a sub-cohort of tumours profiled in a RT-qPCR platform, one to two genes failed in 25% tumours. In our previous work,³³ a 26-gene signature was initially generated from a retrospective cohort of Affymetrix microarray platform and transferred into a customised TaqMan low-density array, where one gene repeatedly failed and was replaced by another gene. Once the gene expressions were reliably measured using the application assay, the corresponding signature could be applied to generate the distribution of tumour signature scores and identify the relevant threshold value (e.g. median) for binarisation into high- or low-hypoxia status. Then, any prospective tumour sample could be directly stratified in an individualised manner.

Another practical consideration is the inherent heterogeneity that exists in tumours. Hypoxia gene signatures reflect the level of hypoxia in a particular biopsy rather than the whole tumour. Reports^{21,53} showed desirably low intratumour heterogeneity associated with two head and neck signatures. However, it may not be possible to generalise this finding as each signature should be analysed individually. Owing to the restriction of resources, the strategy of analysing one biopsy for each tumour is currently used for all hypoxia gene signatures. Whether evaluating multiple

Figure 2. Pros and cons of different categories of hypoxia biomarkers.

	Gene signature	Electrode	Pimonidazole	Imaging	Protein
Gold standard	No	Yes	No	No	No
Invasiveness	No	Yes	No	No	No
Applicability	All tumours	Accessible tumours	All tumours	All tumours	All tumours
Overall cost	Medium	Medium	Medium	High	Low
Sampling bias	High	High	High	Low	High
Transferability across cancers	Low	High	High	High	High
Clinical feasibility	High	Low	Medium	Medium	High

biopsies or applying image-guided biopsy selection will improve the predictive significance of hypoxia signatures requires evaluation in large validation studies.

SUCCESSFUL HYPOXIA GENE EXPRESSION SIGNATURES

Hypoxia gene expression signatures have already shown potential for clinical application. Three gene signatures were validated as predictive biomarkers in retrospective studies of randomised trials of hypoxia-targeting therapies.^{30,31,33} Our 26-gene head and neck hypoxia signature³³ is currently undergoing prospective validation in the UK Phase III NIMRAD trial.⁹ The Danish 15-gene head and neck signature is also being assessed prospectively.⁵³ We are also at the early stage of developing biomarker-driven trials for prospective qualification of our bladder and soft tissue sarcoma signatures. Therefore, hypoxia gene signatures are at the forefront of all the hypoxia biomarkers for progressing to clinical application for personalising treatments.

COMPARISON BETWEEN DIFFERENT HYPOXIA BIOMARKERS

The relative pros and cons of different hypoxia biomarkers are summarised in Figure 2. Using oxygen electrodes is the gold standard approach, but its clinical practicality is limited by its poor availability, invasiveness and applicability to only accessible tumours.⁵⁴ Based on the subjective experience of the senior author of this review paper, the five biomarkers could be ranked according to the overall cost as imaging >electrode ≈ gene signature ≈ pimonidazole >protein marker. The costs have significant implications for biomarker qualification, since proper prospective evaluation usually requires very large sample sizes.⁵⁵ Imaging biomarkers have the advantages of whole tumour assessment and repeatability. Gene signatures have the disadvantage of currently appearing to have limited transferability between tumour types. The other biomarkers are broadly applicable to all cancers, although their hypoxia specificity could be challenged, especially for the protein markers.^{54,56} In terms of clinical feasibility, gene signatures and protein markers are the most convenient as both can be performed on routine diagnostic biopsies. Imaging biomarkers require dedicated equipment and skilled operators. Use of pimonidazole requires drug injection and additional biopsy.^{20,54} Although protein markers are the easiest and cheapest to measure, they suffer from a lack of reliability. Few protein makers have entered the clinical arena. In contrast,

gene expression signatures are more reliable²¹ and several are commercially available.^{57–59}

The choice of hypoxia biomarker will also depend on the context of its use. For some cancers where routine diagnostic biopsies are very small or difficult to obtain, e.g. lung and pancreatic cancers, there are clear advantages in an imaging biomarker. Likewise, an imaging biomarker has the advantage of being suitable as a pharmacodynamic marker of response and when spatial mapping is required. The clinical setting will also be important. Tissue-based biomarkers are attractive in the treatment of primary tumours, but the use of liquid biopsies are more suited to the metastatic setting. There might also be a need to tailor biomarkers to different interventions.

Literature highlights that the agreement between different hypoxia biomarkers is generally weak.^{31,60–63} The discordance could be attributed to intratumour heterogeneity, the dynamic nature of hypoxia and technical limitations. Essentially, this also means that the predictive value is the sole “gold standard” for hypoxia biomarker evaluation.

CONCLUDING REMARKS

Faced with an expanding portfolio of possible agents to combine with radiotherapy and multiple phenotypes that could be measured, there is a need to explore inter-relationships between different biomarkers and interventions. A question of interest is whether hypoxia gene signatures would predict benefit from adding chemotherapy and/or immunotherapy with radiotherapy. For example, there is evidence in the literature that, in

muscle-invasive bladder cancer, only a subset of patients benefit from neo-adjuvant chemotherapy.⁵⁷ Also, response to immunotherapy is usually limited to ~20% of patients, with high toxicity for other patients.^{64,65} As hypoxia might promote resistance to both chemotherapy and immunotherapy,⁵ it will be important to explore whether hypoxia signatures identify patients unlikely to benefit from either chemotherapy or immunotherapy.

Clinical research focuses increasingly on personalised medicine and the identification of biomarkers to guide treatment decisions for individual patients. Amid the growing options of radiotherapy-based multimodality treatments available or being investigated (chemotherapy, hypoxia modification, immunotherapy) for patients in the UK, there is an urgent clinical need for predictive biomarkers. Hypoxia, a widely studied phenotype at both the clinical and molecular levels, is an obvious target for biomarker research. Gene expression signatures are promising candidates for clinical application as hypoxia biomarkers. More research efforts are now needed to be directed towards clinical qualification of existing biomarkers rather than development of new biomarkers.

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