Tumour Proliferation and Imaging Biomarkers

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Imaging biomarkers play a key role in the identification of biological particularities within tumours and therefore are an important component of treatment personalisation in radiotherapy. Imaging techniques such as PET, SPECT, MRI that employ tumour-specific biomarkers already play a critical role in patient stratification towards individualized treatment.

Keywords: biomarkers ; personalized therapy ; patient stratification ; functional imaging

1. Introduction

According to the latest Global Cancer Statistics, lung cancer is the most commonly diagnosed cancer worldwide, in both males and females (11.6% of the total cases) and the leading cause of cancer death (18.4%) [1]. Non-small cell lung cancers (as opposed to small cell lung cancers) account for about 85% of lung cancer cases and encompass adenocarcinomas, squamous cell carcinomas and large-cell undifferentiated carcinomas. Conventional therapies (surgery, chemo-radiotherapy) are being improved with new drugs and targeted agents.

While the latest technological and pharmaceutical developments have increased the therapeutic index in lung cancer, research over the last decade reveals an imperative need to include radiobiological characteristics of cellular and subcellular structures as well as the tumour microenvironment into the big picture of personalised medicine [2]. Hypoxia, proliferation, intrinsic radioresistance, and the presence of cancer stem cells are only a few, but probably the most critical features that require better management to further improve cancer treatment outcomes in general, and lung cancer treatment in particular. However, the primary tumour is not the only entity to confront. Cancer invasion and metastasis poses a therapeutic challenge by broadening the curative needs from local to systemic disease management. In this context, the identification and quantification of circulating tumour cells represent an important undertaking.

Although most of aforementioned tumour characteristics and their impact on tumour control are well known, there is still no clear-cut solution to manage treatment resistance due to high proliferative potential, the presence of cancer stem cells or circulating tumour cells that are indicative of tumour aggressiveness.

In order to tackle the above challenges, one should first identify the hostile features and then target them with the best currently available techniques. In this respect, biomarkers play a key role, as their specific design allows the identification of tumour areas that are prone to treatment resistance, thus leading the way towards personalised, targeted therapies.

2. Tumour Proliferation and Imaging Biomarkers

2.1. Tumour Proliferation

Cellular proliferation is a prerequisite for tissue growth and development. Uncontrolled proliferation is characteristic of cancer cells and represents one of the hallmarks of neoplastic growth. The rate of tumour proliferation differentiates slowly proliferating from rapidly proliferating tumours, a feature that dictates the type of treatment required for tumour control. The evaluation of a tumour’s proliferative ability and of its growth kinetics are therefore critical aspects of cancer management.

Cell proliferation rate is commonly assessed through the presence in the cell nucleus of the Ki-67 monoclonal antibody, during the active phases of the cell cycle. The Ki-67 antibody labels nuclei of proliferating cells, enabling the quantification of the proliferating cell fraction within a tumour [3]. Clinical research over the years has proved Ki-67 proliferation index (or labeling index) to be a biomarker with important prognostic and predictive value in a number of cancers, including lung. The retrospective analysis of three NSCLC cohorts involving about 1500 patients showed that Ki-67 proliferation index is a highly significant and independent predictor of survival in these cancers [4]. An important aspect of the study was the individual assessment of Ki-67 correlation with each histological type of NSCLC. In this respect, the high proliferation index (PI) in adenocarcinomas was significantly associated with a worse prognosis for disease-free survival, whereas in

squamous cell carcinomas the high PI was associated with better overall survival rates (cut-off value for PI of 50%). Treatment outcome among adenocarcinoma patients was further influenced by the administration of adjuvant chemo-radiotherapy, showing that patients with high PI may benefit to a higher extent from adjuvant treatment than those with low PI (cut-off value for PI of 25%).

This study showed the importance of data analysis based on histological characteristics (rather than NSCLC as a group) and the definition/validation of a Ki-67 cut-off value for each histological type of NSCLC. Furthermore, it was suggested that the predictive power of Ki-67 labeling could be enhanced by the concurrent employment of other clinical/pathological parameters as well as imaging biomarkers, which would eventually lead to better patient stratification and treatment optimisation.

Another important factor that controls cellular proliferation in lung cancers (and not only) is the epidermal growth factor receptor (EGFR). The EGFR is a transmembrane glycoprotein receptor of the ErbB family of cell surface tyrosine kinases with a role in regulating cell proliferation and apoptosis through signal transduction pathways [5]. Mutations and truncations of its extracellular matrix leads to upregulation of EGFR in several cancers, including NSCLC. Malignant as well as premalignant lesions can overexpress EGFR, with 40–80% of NSCLC patients being identified with abnormal expressions of EGFR (increased gene copy number per cell), with the highest rates seen in squamous cell carcinomas [6][7]. EGFR expression was found to be a poor prognostic factor in NSCLC, requiring efficient anti-EGFR therapies [6]. To date, EGFR-targeted therapies based on tyrosine kinase inhibitors (gefitinib, erlotinib) and monoclonal antibodies (cetuximab) have been developed with limited success, due to acquired or inherent resistance to EGFR inhibition [8]. Next to EGFR, ALK (anaplastic lymphoma kinase) translocations are known to be oncogenic drivers in NSCLC [9]. ALK translocation is associated with high sensitivity to ALK inhibitors such as crizotinib, ceritinib and alectinib [10]. Moreover, the set of mutations in these cancers is much wider. Regarding targeting avenues, ROS1 translocation is associated with a positive response to crizotinib therapy, while for BRAF mutations the combined administration of dabrafenib and trametinib, as well as the low molecular weight tyrosine kinase inhibitors vemurafenib and dabrafenib, were shown to be effective. MET mutations in lung cancer are considered to be predictors of susceptibility to the MET inhibitor crizotinib, whereas RET translocations are correlated with a positive response to targeted therapy with RET inhibitors such as cabozatinib, vandetinib, and alectinib [10]. All these mutations are important therapeutic targets, which can be identified not only in biopsy samples (given that 30% of tumour biopsies yield inadequate tissue for molecular subtyping) but also in cell-free circulating tumour DNA [11].

More recently, research into tumour proliferation has been linked to microRNAs, owing to their role in multiple biological processes, including gene regulation [12]. MicroRNAs (miRNA) are short noncoding RNAs consisting of 21–25 nucleotides that can inhibit translation of messenger RNA (mRNA) and promote mRNA degradation, thus functioning as endogenous negative gene regulators. Through posttranscriptional regulation of gene expression, miRNAs have a great impact on a number of oncogenic pathways. Recent studies demonstrated a relationship between the EGFR signaling pathway and miRNAs, showing a direct regulatory effect on EGFR [13]. Studies in NSCLC revealed the potential of miRNAs to serve in patient stratification (by risk and histology) while also predicting prognosis in early-stage NSCLC [14].

2.2. Imaging Biomarkers for Proliferation

Cellular kinetic parameters are important indicators of tumour proliferation before, during and after therapy, thus their quantification warrants special consideration. As shown above, the most studied proliferation markers and, consequently, the most targeted molecules related to cellular proliferation in lung cancer imaging are EGFR and Ki-67. In this regard, numerous tracers have been developed and trialed with various results [15].

2.2.1. Positron Emission Tomography (PET) Imaging Biomarkers

Fluorodeoxyglucose-F18 (18F-FDG) is the most commonly used PET imaging radiotracer, being an indicator of tumour activity via glucose metabolism, and has an established role in tumour staging and treatment response monitoring. Its role in the assessment of tumour proliferation was also researched, with a considerable number of studies examining the potential of 18F-FDG in predicting EGFR mutation status in NSCLC patients. In a retrospective clinical study involving 109 NSCLC patients, Chen et al. showed that EGFR mutation decreases cellular accumulation of FDG via the NOX4/ROS/GLUT1 axis [16]. The SUVmax values in the cohort with EGFR mutations were significantly lower (6.52 mean value) than in the wild-type EGFR cohort (9.37 mean value, $p < 0.001$). Similarly, in a study of 102 NSCLC patients with EGFR mutation (22%), KRAS mutation (27%) and wild-type profiles (51%), it was observed that 18F-FDG uptake was significantly higher in those harbouring KRAS mutations as compared to EGFR+ or wild-type (SUVmean 9.5 vs. 5.7 vs. 6.6, $p < 0.001$) [17]. These findings are corroborated by a much larger study, encompassing 849 NSCLC patients with

45.9% identified with EGFR mutation, that also showed low SUVmax association with EGFR mutation status [18]. This result could be combined with other clinical factors to improve patient stratification, particularly when EGFR testing is not available [18][19].

A recent study reported on the development of a new PET tracer with high specificity to activating EGFR mutant kinase showing significant correlation between tracer uptake and the EGFR mutation status in both preclinical animal models and in patients with NSCLC [20]. The study aimed to identify, via a new imaging tracer—18F-MPG (*N*-(3-chloro-4-fluorophenyl)-7-(2-(2-(2-(2-^{18}F-fluoroethoxy) ethoxy) ethoxy) ethoxy)-6-methoxyquinazolin-4-amine)—, those patients that are sensitive to EGFR-TKIs and to monitor the efficiency of EGFR-TKI therapy. The cut-off value for SUVmax was set at 2.23, showing a greater response to EGFR-TKI in those presenting with SUVmax ≥2.23 as compared to patients with values <2.23 (81.58% vs. 6.06%). Furthermore, 18F-MPG uptake positively correlated with median progression-free survival [20].

While 18F-FDG has its own merits in the functional imaging of lung cancer, it is not the optimal indicator of proliferation, showing poorer correlation with cellular proliferation markers than other PET tracers. Fluoro-3′-deoxythymidine-F18 (18F-FLT), a successfully used imaging marker of cellular proliferation, is a radiolabeled structural analog of a DNA nucleoside—thymidine—and its uptake relates to the activity of thymidine kinase 1 (TK1) that is expressed during DNA synthesis in the S-phase of the cell cycle [21]. The uptake of 18F-FLT in tumour cells is lower as compared to 18F-FDG, as it only accumulates in cells during the S-phase [15]. Yet, several studies demonstrated the superior correlation of 18F-FLT with cellular proliferation markers when compared to the traditional 18F-FDG [22][23]. In one of the first comparative studies that involved a cohort of 26 lung cancer patients, Buck et al. showed high correlation between 18F-FLT uptake and Ki-67 index ($p < 0.0001$; r = 0.92), and concluded that 18F-FLT may be a better imaging marker than FDG for response assessment and outcome prediction [22]. These observations are supported by a recent meta-analysis that assessed 1213 patients from 22 imaging studies that correlated the Ki-67 labeling index with FDG and FLT uptake, respectively, showing that the latter is a more robust marker of tumour proliferation in lung cancer [23].

In a recent pilot study, Kairemo et al. demonstrated the feasibility of 18F-FLT PET in monitoring treatment response by early signal activity in NSCLC patients receiving targeted therapies (c-MET inhibitors) [24]. Several others have confirmed the potential of 18F-FLT PET imaging to monitor and guide molecular targeted therapies in NSCLC [25][26][27].

Next to the most common Fluor-based radiotracers employed in PET for tumour proliferation imaging, copper is another successful candidate. Functional imaging with PET employing 64Cu-ATSM (Cu-labeled diacetyl-bis(N(4)-methylthiosemicarbazone) and 18F-FDG was undertaken for the intratumoral distribution assessment of the two radionuclides in Lewis lung carcinoma tumour cells implanted in mice [28]. Both proliferation markers (Ki-67 and BrdU-bromodeoxyuridine) and the hypoxic marker, pimonidazole, were used to compare radionuclide uptake with immunohistochemical staining patterns. The association of staining with radionuclide accumulation revealed an increase in Ki-67 positive areas with 18F-FDG uptake increase and, at the same time, a decrease with 64Cu-ATSM accumulation. Conversely, the other proliferation marker, BrdU, showed an opposite behaviour, with the number of BrdU-positive cells being positively correlated with 64Cu-ATSM uptake and negatively related to 18F-FDG accumulation. Given that BrdU is a marker for proliferation by way of DNA synthesis, the fact that cells with high 64Cu-ATSM uptake positively correlated with the number of BrdU cells indicates that they are able to undergo DNA synthesis, though not during the proliferation process (denoted by the low Ki-67 levels which are specific to G1 and early S phase). This result suggests that cells in regions with high 64Cu-ATSM uptake were quiescent, yet sustained DNA synthesis and were sensitive to progression factors, just like quiescent cancer stem cells. Clonogenic assays within the same study have proven the stem-like properties of cells originating from high 64Cu-ATSM uptake tumour areas [28]. Furthermore, pimonidazole-positive areas were specific to regions with low 64Cu-ATSM accumulation, suggestive of mild hypoxic conditions, still optimal for the thriving of clonogenic tumour cells.

This study is a clear illustration of the complexity of the tumour microenvironment and of the many factors that influence tumour development and response to therapy (hypoxia, proliferation, cancer stem cells). Based on the above results, 64Cu-ATSM could potentially serve as a complex imaging biomarker to supply prognostic information for treatment adaptation and optimisation.

2.2.2. Single Photon Emission Computed Tomography (SPECT) Imaging Biomarkers

Beside PET tracers, a number of researchers attempted to develop SPECT radioisotopes for novel insights into EGFR targeting. The capacity of 99mTc-HYNIC-MPG ((2-(2-(2-(2-(4-(3-chloro-4- fluorophenylamino)-6-methoxyquinazolin-7-yloxy)ethoxy)ethoxy)ethoxy)ethyl-6-hydrazinylnicotinate hydrochloride) was evaluated in detecting EGFR-activating mutations both in vitro and in vivo, using human NSCLC cell lines [29]. The study showed that of the four cell lines

(EGFR+, EGFR− and wild-type), 99mTc-HYNIC-MPG uptake was the highest in the cell line with exon 19 deletion (PC9), probably due to the activating mutations in EGFR tyrosine kinase domain. The results could serve to further stratify NSCLC patients by identifying the subgroup that would benefit the most from targeted therapies with EGFR-TKIs [29].

Table 1 is a compilation of different functional imaging agents tested as markers for tumour proliferation in NSCLC.

Table 1. Functional imaging biomarkers for tumour proliferation in non-small cell lung cancer (NSCLC).

Study Aim [Ref]	**Study Type**	**Proliferation Marker/ Targeting Agent**	**Comments**
Positron Emission Tomography			
Proliferation imaging with ^{18}F-FLT vs ^{18}F-FDG [Buck et al. 2003] [22]	Prospective study (26 patients with pulmonary nodules)	**Proliferation marker:** Ki-67 **Targeting agent:** ^{18}F-FLT ^{18}F-FDG	A highly significant correlation (p < 0.0001) and a high correlation coefficient (r = 0.92) was observed between ^{18}F-FLT uptake and Ki-67 index, while the correlation coefficient between Ki-67 and ^{18}F-FDG was weak (r = 0.59). No FLT uptake was detected in non-proliferating tumours.
PET imaging for EGFR mutation evaluation and response to treatment [Sun et al. 2018] [20]	Preclinical rodent model; Clinical NSCLC study	**Proliferation marker:** EGFR **Targeting agent:** ^{18}F-MPG	A greater response to EGFR-TKI was found in patients with SUV_{max} ³ 2.23 (81.58% vs 6.06%). Median progression-free survival was also longer (348 days) in the cohort with SUV_{max} ³ 2.23 than in SUV_{max} < 2.23 (183 days). ^{18}F-MPG PET for quantification of EGFR-activating mutation status could identify patients sensitive to EGFR-TKIs.
Evaluation of the role of ^{64}Cu-ATSM in PET imaging *[Oh* et al. *2009]* [28]	In vivo mice study (Lewis lung carcinoma tumour cells implanted in mice)	**Proliferation markers:** Ki-67 BrdU **Targeting agent:** ^{64}Cu-ATSM ^{18}F-FDG	Tumour regions with high ^{18}F-FDG but low ^{64}Cu-ATSM uptake correlated with increase in Ki-67. On the other hand, the number of BrdU-positive cells were positively correlated with ^{64}Cu-ATSM uptake and negatively related to ^{18}F-FDG accumulation. This suggests that cells in regions with high ^{64}Cu-ATSM uptake were quiescent, yet were sensitive to progression factors, like quiescent CSCs.
Single Photon Emission Computed Tomography			
Evaluation of **^{99m}Tc-HYNIC-MPG for detection of EGFR-activating mutations** [Xiao et al. 2017] [29]	In vitro cell line study (human NSCLC cell lines EGFR+/- and wild-type); In vivo animal xenograft model	**Proliferation marker:** EGFR **Targeting agent:** **^{99m}Tc-HYNIC-MPG**	**^{99m}Tc-HYNIC-MPG uptake was the highest in the cell line with exon 19 deletion (PC9), probably due to the activating mutations in EGFR tyrosine kinase domain.** SPECT imaging with ^{99m}Tc-HYNIC-MPG could potentially identify NSCLC patients that would benefit the most from targeted therapies with EGFR-TKIs.

Magnetic Resonance Imaging			
EGFR targeting with active iron oxide NP for MRI [Wang et al. 2017] [30]	H460 lung cancer cells (in vitro) and tumour-bearing rats (H460 lung xenografts) in vivo.	**Proliferation marker:** EGFR **Targeting agent:** Anti-EGFR-polyethylene glycol-superparamagnetic iron oxide (anti-EGFR-PEG-SPIO)	Both in vitro and in vivo MRI studies showed the potential of anti-EGFR-labeled iron oxide nanoparticles to identify and target lung cells that overexpress EGFR. The study had both imaging and therapeutic (theranostic) goals achieved with anti-EGFR targeting based on magnetic nanoparticles using MRI and focused ultrasound ablation.

2.2.3. Magnetic Resonance Imaging (MRI) Biomarkers

The latest advances in biomaterials, specifically in nanomedicine, have greatly increased the sensitivity of imaging techniques using magnetic resonance to perform accurate and non-invasive functional imaging. In this regard, one of the recent developments is in the field of superparamagnetic iron oxide (SPIO) nanoparticles (40–50 nm), whereby polyethylene glycol-coated SPIO nanoparticles (PEG-SPIO) were synthesized and further labeled with high affinity anti-EGFR monoclonal antibody (cetuximab) for targeted delivery to lung cancer that overexpresses EGFR [30]. The targeting efficiency, MRI contrast enhancement and cytotoxicity of this nanocomposite was evaluated in both H460 lung cancer cells (in vitro) and tumour-bearing rats (H460 lung xenografts) in vivo. The uptake of the nanocomposite in the cell lines was evaluated by Prussian blue staining which showed an increased cellular uptake of anti-EGFR targeted NPs compared to non-targeting NPs at the same iron concentration, suggesting that the high cellular accumulation of anti-EFGR NPs is due to the EGFR receptor-mediated endocytosis pathway. This was also illustrated by TEM (transmission electron microscopy) imaging, where cells incubated with anti-EGFR targeting NPs showed the presence of electron-dense particles in the cell endosome, in contrast with those incubated with non-targeting NPs, which showed no such uptake. To further confirm these results, MRI-based investigation was undertaken by measuring the T2 weighted signal intensity of lung cells after incubation with NPs having various iron concentrations. It was observed that the T2 signal decreased with the increasing iron concentrations in the EGFR targeting NPs group. Furthermore, the signal intensity of lung cancer cells that overexpressed EGFR and were targeted with anti-EGFR-PEG-SPIO decreased more significantly than in the PEG-SPIO (non-targeting NPs) group. The study concluded that efficient identification and targeting of lung cells overexpressing EGFR can be achieved by means of anti-EGFR-PEG-SPIO nanocomposite, under MRI monitoring [30].

2.3. Summary of Current Status for Proliferation Biomarkers

While Ki-67 is a marker of proliferation that is well studied in lung cancer, EGFR has a less clear impact and its prognostic role is obscured by new therapies currently employed in clinical practice (such as EGFR-TKIs). To justify further developments in the field of new tracers for EGFR positive NSCLC, also considering the rapid pace of treatment evolution in this subset of patients, a cost–benefit analysis would help clinicians in their decision making. While there are some promising reports, neither the treatment response prediction nor the prognosis of EGFR tumours offered by these biomarkers are convincing enough to support wide clinical implementation.

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