

Experimental results and related clinical implications of PET detection of epidermal growth factor receptor (EGFr) in cancer

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The epidermal growth factor receptor (EGFr) is one of the most studied molecules as a target for cancer therapy. Over these last few years, several studies attempting to identify predictive biomarkers of treatment response, such as the receptor status or other molecules related to the downstream signalling pathway, have been conducted. However, from a clinical point of view, the information obtained from *ex vivo* analyses still has various limitations that may be overcome by the combination with molecular imaging technologies which may provide a noninvasive, global, *in vivo* evaluation of the molecular tumour background. The aim of this review is to report the preclinical results of all positron emission tomography (PET) tracers synthesized until now for *in vivo* detection of EGFr in cancer. Two classes of PET compounds have been developed: labelled small molecules such as tyrosine kinase inhibitors and labelled monoclonal antibodies. The *in vitro* and *in vivo* results of these PET tracers are very different depending on the chemical properties, positron emission radionuclide, or animal models. As a consequence, various critical questions are still open, and the implications of a translation in the clinical setting for EGFr imaging in cancer patients is discussed.

Key words: colon cancer, epidermal growth factor receptor (EGFr), lung cancer, monoclonal antibodies, PET, tyrosine kinase inhibitors

introduction

The epidermal growth factor receptor (EGFr) is a receptor with tyrosine kinase (TK) activity that belongs to *c-erb* family [1]. The receptor is divided in three regions: an extracellular region with a ligand-binding domain, a trans-membrane region and cytoplasmic region with a TK domain. Several proteins are known as ligand of EGFr (EGF, transforming growth factor- α , neuregulins). The binding between a ligand and the extracellular domain causes the receptor's dimerisation and consequently the phosphorylation of the tyrosine residues, activating various downstream signalling pathways [2]. The aberrant EGFr pathway activation may be the results of different molecular abnormalities which include gene amplification, receptor mutations, growth factors overexpression or cross-link downstream signalling pathways activation [3]. The biological role of EGFr in oncology is crucial because its deregulation is involved in several tumorigenic processes: uncontrolled cell proliferation, inhibition of apoptosis, angiogenesis and metastasis potential [4]. EGFr has

been shown to be differentially deregulated, overexpressed, mutated or amplified in many types of cancer [5, 6]. It may be associated to a more aggressive disease, therapy resistance, shorter survival [7]. Two major strategies for inhibiting EGFr pathway have been developed: anti-receptor monoclonal antibodies, such as cetuximab and panitumumab, or small molecular inhibitors of intracellular TK such as gefitinib and erlotinib [8]. The clinical applications of these drugs are several and the results are different and sometimes contradictory depending on types of tumours and clinical setting.

However, despite the large clinical trials and the wide use of anti-EGFr drugs in practice, some clinical questions are still open. The assessment of EGFr in *ex vivo* tumours specimens is still controversial for both methodological and biological reasons [9]. EGFr was evaluated by immunohistochemistry (IHC) in most clinical studies and in clinical practice, but it is now well known that IHC is not an ideal method for EGFr detection for several factors [10]. Molecular studies of the receptor including RT-PCR for the RNA gene expression, ELISA or western blotting for the protein level quantification, FISH for gene amplification still need to be validated. At the moment, there are no standardised methods for EGFr status available for everyday practice and consequently no clear predictive role of response to anti-EGFr drugs has been found

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in large series [9]. The recent accumulating data on other surrogate markers for treatment prediction have improved the knowledge for selecting patients, but most of the mechanisms of intrinsic and acquired resistance still need to be better elucidated [11]. Therefore, even though EGFr is widely recognised as the target of the new drugs in oncology, no conclusive data are available on the EGFr axis in cancer and its inhibition.

Taking into account these considerations, the *in vivo* identification and distribution of EGFr may be a novel and potential tool for globally assessing the receptor. Different imaging approaches have been developed for specifically detecting EGFr from optical imaging modalities to single photon emission computed tomography and positron emission tomography (PET) technologies [12]. The aim of this review is to discuss the clinical implications of the *in vivo* EGFr detection in medical oncology and to report the experimental results already available on the development of new PET bioprobes.

clinical implications of *in vivo* EGFr imaging

The most intriguing and relevant clinical application of PET detection of EGFr in medical oncology is the potential predictive role for optimising the selection of patients who are likely to benefit from EGFr inhibitors treatments and for monitoring the treatment's efficacy. In the last few years, several predictive biomarkers have been studied. In order to focus and discuss the real impact of *in vivo* evaluation of EGFr in cancer patients, it is necessary to examine the current state of the art on EGFr inhibitors' predictive markers especially in non-small-cell lung cancer (NSCLC) and colorectal cancer (CRC).

lung cancer

Gefitinib and erlotinib have been approved for the treatment of metastatic NSCLC after failure of conventional chemotherapy [8]. The first trials investigating the efficacy of gefitinib and erlotinib administration showed a dramatic response in some cases [13–19]. In particular, the most responsive patients are female, Asian and have never smoked and patients with a histologic adenocarcinoma tumour type. Regarding molecular markers, EGFr expression, EGFr gene copy number, EGFr mutational status, p-Akt associated to the EGFr signalling pathway were the most and best investigated (Table 1).

EGFr expression determined by IHC showed controversial results for predicting EGFr-TK inhibitors response [20–25]. In general, the protein expression is not considered to be useful in lung cancer [8, 26]. These results may be mostly explained by the inadequacy of IHC in testing the EGFr status [10]. Increased EGFr gene copy number, detected by FISH, was widely studied over the few last years and it is actually considered a sensitive predictor factor to TK inhibitors [24, 25, 27–29]. Cappuzzo and colleagues were the first to study the correlation between clinical outcome and the EGFr status with FISH in patients with advanced NSCLC treated with gefitinib. They showed that patients with positive FISH tumours have a significantly higher response rate (RR), longer time to progression (TTP) and 1-year survival rate as opposed to those with a negative FISH [24]. The same analysis was used in other studies showing that the increased gene copy number is associated to higher RR and

longer survival after gefitinib or erlotinib treatments in comparison to placebo [23, 25, 27, 28]. More recently, Cappuzzo et al. [29] reported a prospective trial to evaluate gefitinib sensitivity in NSCLC patients considering smoking history, EGFr gene copy number assessed by FISH and p-Akt status. Moreover, they studied the mutational status of EGFr, HER-2 and K-ras. They found that patients with an EGFr FISH-positive tumour had a better RR and longer TTP and survival and they concluded that EGFr FISH analysis is an appropriate predictor factor to gefitinib sensitivity [29]. On the contrary, other methods than FISH for assessing the gene copy number have been used but the results are controversial [26].

Different somatic gain-of-function mutations of TK domain, within exon 18–24, where the TK inhibitors compete to adenosine triphosphate (ATP) are involved in EGFr inhibitors' responsiveness [6]. The most common points of mutation associated with gefitinib response are located in the exon 19 with an in-frame deletion in codons 746–750, and the other one in exon 21 with a missense mutation L858R that led to change leucine 858 with arginine in the EGFr activation loop [30, 31]. Since the first evidence, several retrospective studies confirmed the predictive role of EGFr mutations of treatment efficacy in NSCLC. Moreover, EGFr mutations are most frequent in responsive patients with one or more clinical sensitive characteristics such as histological features of adenocarcinoma, female, Asian, a nonsmoking history [31–34].

Until now, few prospective trials investigated the correlation between mutations and RR in previously untreated patients with advanced NSCLC [35–37]. However, the predictive significance seems to be different according to the type of mutation [38–40]. Even though these accumulating data demonstrated the role of EGFr mutations in responsiveness to TK inhibitors, a role as prognostic factor has also been supposed. Survival advantage in patients with EGFr mutant tumours has been observed in comparison to EGFr nonmutant tumours among patients treated with chemotherapy alone [41, 42].

With regard to other biomarkers, the activation of Akt pathway, as showed by Cappuzzo et al. [43], correlates with a better RR, disease control rate (DCR) and TTP in patients with NSCLC treated with gefitinib.

K-ras plays a key role in the RAS/MAPK pathway downstream signalling of EGFr and other growth factor receptors. In lung cancer, K-ras abnormalities seem to be associated with a history of cigarette use [44, 45]. K-ras mutations are associated with resistance to gefitinib or erlotinib [46, 47].

HER-2 gene amplification in lung cancer studied with FISH is associated to higher RR, longer TTP and survival in patients treated with gefitinib [48].

In conclusion, EGFr mutations increased EGFr gene copy number and never smoking history are mostly associated with the treatment's sensitiveness. However, the incidence of mutant EGFr tumours is low. Not all patients with mutations respond to TK inhibitors. Moreover, most TK inhibitors' responsive mutant NSCLC become resistant to therapy. Various mechanisms of acquired resistance have been studied [49, 50]. A mutation in exon 19 T790M is responsible for secondary resistance to TK inhibitors in ~50% [51–53]. More recently, genome-wide copy number analysis and mRNA expression profiling in cell lines which have developed gefitinib

Table 1. Major predictive factors of response to gefitinib and erlotinib in lung cancer

Marker	Authors	Years	No. of patients	Method	Drug	Responders versus Nonresponders	Predictive value	Other markers evaluated	Ref.	
epidermal growth factor receptor (EGFr)	EGFr protein expression	Cappuzzo	2003	43	Immunohistochemistry	Gefitinib	–	No	HER2 expression	[20]
		Parra	2004	50	Immunohistochemistry	Gefitinib	$P = 0.108$	No	–	[21]
		Villaflor	2006	87	Immunohistochemistry	Gefitinib	–	No	p-Akt protein expression EGFr mutations, EGFr gene copy number, C7 gene copy number	[22]
	Cappuzzo	2005	98	Immunohistochemistry	Gefitinib	$P = 0.03$	Yes (response)	EGFr gene copy number, EGFr mutations, Akt activation	[24]	
	Hirsch	2007	200	Immunohistochemistry	Gefitinib	$P = 0.002$ $P < 0.001$ $P = 0.003$	Yes (response) Disease control rate Overall survival	p-Akt protein expression, EGFr/HER2 gene copy number, EGFr mutations, KRAS mutations	[25]	
EGFr gene copy number	Cappuzzo	2005	102	FISH	Gefitinib	$P < 0.001$	Yes (response)	EGFr protein expression, EGFr mutations, Akt activation	[24]	
	Tsao	2005	221	FISH	Erlotinib	$P = 0.03$	Yes (response)	EGFr protein expression, EGFr mutations	[23]	
	Hirsch	2006	370	FISH	Gefitinib	$P = 0.045$	Yes (survival)	EGFr protein expression, p-Akt protein expression, EGFr mutations, KRAS mutations, BRAF mutations	[27]	
	Hirsch	2005	81	FISH	Gefitinib	$P = 0.042$	Yes (survival)	HER2 gene copy number	[28]	
	Hirsch	2007	183	FISH	Gefitinib	$P < 0.001$ $P < 0.001$ $P = 0.002$	Yes (response) Disease control rate Overall survival	EGFr protein expression, HER2 gene copy number, EGFr mutations, Akt expression, KRAS mutations	[25]	
	Cappuzzo	2007	42	FISH	Gefitinib	$P < 0.001$ $P < 0.02$	Yes response Time to progression	p-Akt protein expression, EGFr protein expression, HER2 gene copy number, EGFr mutations, HER2 mutations, KRAS mutations	[29]	

Table 1. (Continued)

Marker	Authors	Years	No. of patients	Method	Drug	Responders versus Nonresponders	Predictive value	Other markers evaluated	Ref.
EGFr gene mutations	Lynch	2004	25	Amplification and sequencing	Gefitinib	$P < 0.001$	Yes (response)	–	[30]
	Paez	2004	119	Amplification and sequencing	Gefitinib	$P = 0.0027$	Yes (response)	–	[31]
	Pao	2004	18	Amplification and sequencing	Gefitinib	$P = 0.004$	Yes (response)	–	[32]
			17		erlotinib	$P = 0.003$			
	Paz-Ares	2006	43	GenScan, TaqMan assay and sequencing	Erlotinib	$P = 0.038$	Yes (response)	–	[35]
	Inoue	2006	16	Amplification and sequencing	Gefitinib	–	–	–	[36]
	Sequist	2008	34	Amplification and sequencing	Gefitinib	–	–	EGFr gene copy number	[37]
Tsao	2005	197	PCR assays with the use of AmpliTaq Gold	Erlotinib	–	No (survival)	EGFr protein expression, EGFr gene copy number	[23]	
Exon 19 deletion versus exon 21 L858R point mutation	Riely	2006	34	Nondirect sequencing PCR methods	Gefitinib erlotinib	$P = 0.01$	Yes (survival)	–	[38]
	Hirsch	2006	157	Amplification and sequencing	Gefitinib	$P = 0.02$	Yes (response)	–	[40]
	Jackman	2006	36	Amplification and sequencing	Gefitinib erlotinib	$P = 0.04$	Yes (survival)	–	[39]
Other markers than EGFr									
Akt pathway KRAS gene mutation	Cappuzzo	2004	106	Immunohistochemistry	Gefitinib	$P = 0.003$	Yes (response)	P-MAPK Immunohistochemistry	[43]
	Pao	2005	60	PCR with the HotStarTaq Master Mix Kit and sequencing reactions	Gefitinib	$P = 0.0373$	Yes (resistance)	EGFr mutations	[46]
					erlotinib	$P = 0.5531$			
Tsao	2006	206	PCR amplification and direct bidirectional sequencing of the PCR product	Erlotinib	$P = 0.03$	Yes (resistance)	EGFr mutations	[47]	
HER 2 gene copy number	Cappuzzo	2005	102	FISH	Gefitinib	$P = 0.001$	Yes (response)	HER2 protein expression, EGFr protein expression, EGFr gene copy number, EGFr mutations	[48]

resistance showed a MET proto-oncogene amplification [54]. Moreover, in 18 lung cancer that were initially responsive and then become resistant to TK inhibitors, MET amplification was positive in four cases (22%) [54].

The acquisition of resistance mechanisms may occur during the natural history of metastatic disease and we are not always able to detect the new biological abnormalities because it requires invasive procedures. The activation status of EGFr signalling pathway may be detected by *in vivo* imaging using PET probes specifically targeting the EGFr phosphorylated status [55]. This approach is noninvasive and is repeatable in different phases of a metastatic disease.

colorectal cancer

The predictive factors of response to anti-EGFr monoclonal antibodies, such as cetuximab and panitumumab both approved for the treatment of advanced CRC, may be clinical such as the occurrence of skin rash after treatment or biological such as the receptor's status and the molecules of EGFr signalling pathway (Table 2).

As well known, even if EGFr represents the main target of these drugs, the role of EGFr status as a predictive marker of response is unclear and controversial. Unlike the powerful predictive role of HER-2 in breast cancer, the data available from clinical trials indicate that, also in CRC, there is no relationship between the EGFr expression detected by IHC and the response to anti-EGFr treatments [56–58]. Regarding EGFr gene copy number, the available data are also discordant. The first evidence that EGFr gene copy number assessed by FISH may identify patients with metastatic CRC who are likely to respond to cetuximab and panitumumab was reported by Moroni et al. [59], but the number of patients was very small and no cut-off for discriminating EGFr FISH-positive versus EGFr FISH-negative patients was proposed. This trend was confirmed by subsequent studies, even if methods of tissue processing and cut-off scoring systems discriminating responders' and not nonresponders' tumours were not standardised between these studies, as a consequence the results are not always homogenous [60–63]. In a large series of 346 patients, EGFr gene copy number assessed by quantitative PCR did not correlate with tumour response or progression-free survival (PFS) but only with survival, indicating that EGFr gene copy number may be an independent prognostic factor and not necessarily predictive factor of response to cetuximab [64]. More recently, Italiano et al. [65] reported that EGFr gene copy number as detected by FISH is not statistically correlated with objective RR, DCR, PFS and survival. No somatic EGFr gene mutations have been demonstrated to have clinical implications in CRC [64, 66]. Genetic polymorphisms of EGF and EGFr were also studied and a genetic variant of EGFr intron-1 and EGF 61A>G was found to be associated with PFS and survival of patients treated with cetuximab [67]. Finally, the activation status of the receptor and not the receptor at all, supposed to be related to carcinogenesis, was also proposed as predictive factor [67, 68]. In a very small series, high phosphorylated EGFr immunohistochemical score was reported to be correlated with a trend of higher disease control in patients treated with cetuximab [69]. The lack of a significant and clear correlation between the EGFr status and tumour response to cetuximab and panitumumab led to the

investigation of other markers than the receptor. The one most studied is the assessment of K-ras mutational status. K-ras mutations induce a constitutive activation of the downstream signalling pathway; therefore, it might be associated with resistance to anti-EGFr drugs independently of the EGFr tumour expression level. The first clinical evidence that K-ras mutations could be predictive of resistance to cetuximab-based therapy in advanced CRC was demonstrated by Lièvre et al. in 2006 (63). In a pooled analysis considering also Moroni's previous results, they found that the probability to have no response to cetuximab was 91.3% in the presence of K-ras mutation and the probability to have a response was 50% when no K-ras mutation was identified. Furthermore, the survival of K-ras wild-type patients was significantly longer in comparison with K-ras-mutated patients. The acquisition of resistance to cetuximab induced by K-ras mutations was supported by several recent studies [70–79]. In all these studies, regardless of the method used, the presence of K-ras mutations was highly associated with cetuximab resistance and the difference in terms of overall RR and survival was statistically significant. Furthermore, wild-type K-ras status may confer sensitivity also to panitumumab and could be considered in selecting patients with metastatic CRC as candidates for this treatment [80]. However, a recent pre-clinical study assessed on 22 colon cancer cell lines has shown different and not homogenous correlation between RAS and/or BRAF mutational status and response to cetuximab [81]. In addition to K-ras gene status, some studies investigated other molecular factors, such as BRAF, PTEN/PIK3CA and NF- κ B, involved in EGFr downstream signalling pathway and other tumour levels such as Cox-2, IL-8 and VEGF [63, 72, 74, 81–83]. In a small series, the loss of PTEN protein expression in at least 50% of tumour cells by IHC seemed to be associated with nonresponsiveness to cetuximab: 62% of patients with intact protein expression had an objective response to cetuximab-based therapy, whereas 0% of patients with loss of PTEN protein expression had benefit from treatment [72]. Combining the loss of PTEN protein expression with EGFr and K-ras gene status, the authors proposed an algorithm for predicting response to cetuximab, indicating that the mechanisms of resistance are multiple and cross-talking with each other. Since one of the supposed mechanism of action of cetuximab is mediated through the antibody-dependent cell-mediated cytotoxicity (ADCC), Zhang et al. [84] demonstrated that two gene polymorphisms of fragment c gamma receptors (FCGR2A-H131R and FCGR3A-V158F) involved in ADCC are also associated with shorter PFS in advanced CRC patients treated with cetuximab. However, these data need to be confirmed and more further investigated because the exact role of ADCC in metastatic CRC patients is still uncertain. Finally, a correlation between the reduction of VEGF levels of at least 50% and tumour response to cetuximab, TTP and survival was found in a small series of advanced CRC patients, indicating that VEGF modifications may be potentially useful to monitor the response to EGFr inhibitors [85].

To summarise, over these last few years, many biological factors in CRC have been investigated for their predictive role of treatment response, but these studies have not shown always clear and conclusive data. Some clinical questions are still open: some of these factors probably have only a prognostic

Table 2. Predictive factors of response to cetuximab and panitumumab in colorectal cancer

Marker	Authors	Years	No. of patients	Method	Drug	Responders versus Nonresponders	Predictive value	Other markers evaluated	Ref.
Epidermal growth factor receptor (EGFr)									
EGFr protein expression	Cunningham	2004	329	Immunohistochemistry	Cetuximab		No	–	[57]
	Saltz	2004	57	Immunohistochemistry	Cetuximab		No	–	
	Chung	2005	16	Immunohistochemistry	Cetuximab		No	–	[58]
	Moroni	2005	31	FISH	Cetuximab	$P < 0.0001$	Yes (response)	PIK3CA, KRAS, BRAF mutations	[59]
	Lenz	2006	346	Amplification and sequencing	Cetuximab		No	EGFr gene mutations	[64]
	Italiano	2007	47	FISH	Cetuximab	$P < 0.68$	No	EGFr protein expression	[65]
	Sartore-Bianchi	2007	58	FISH	Panitumumab	$P < 0.0009$ (cut-off < 2.47 per nucleus)	Yes (response)	–	[60]
						$P < 0.0007$ (cut-off < 43%)			
	Cappuzzo	2008	85	FISH	Cetuximab	$P < 0.0001$	Yes (response)	–	[62]
	Vallbohmer	2005	39	Real-time PCR	Cetuximab	$P < 0.65$	No	CCND1, Cox-2, IL-8, VEGF expression levels	[83]
EGFr gene mutations	Lenz	2006	346	Amplification and sequencing	Cetuximab		No		[64]
Phosphorylated EGFr	Personeni	2005	23	Immunohistochemistry	Cetuximab	$P < 0.05$	Yes (response)	–	[69]
EGFr polymorphisms	Graziano	2008	110	Amplification and sequencing	Cetuximab	$P = 0.05$	Yes (response)	EGF polymorphisms	[67]
Other markers than EGFr									
KRAS gene mutation	Lievre	2006	30	Direct sequencing	Cetuximab	$P < 0.0003$	Yes (resistance)	BRAF, PIK3CA mutations	[63]
	Di Fiore	2007	59	Direct sequencing PCR-LCR SNaPshot	Cetuximab	$P < 0.0005$	Yes (resistance)	–	[71]
	Khambata-Ford	2007	110	Array analysis	Cetuximab	$P < 0.0003$	Yes (resistance)	Epiregulin and amphiregulin gene expression	[70]
	Lievre	2008	114	ABI 7900HT Sequence detection System	Cetuximab	$P < 0.001$	Yes (resistance)	–	[73]
	De Roock	2008	113	7500HT real-time PCR	Cetuximab	$P < 0.0000001$	Yes (resistance)	BRAF mutations	[74]
	Amado	2008	427	Allele-specific real-time PCR	Panitumumab		Yes (resistance)	–	[80]
PTEN expression	Frattini	2007	27	Immunohistochemistry	Cetuximab	$P < 0.001$	Yes (resistance)	EGFr protein expression, EGFr gene copy number, KRAS mutations	[72]
NF-kB	Scartozzi	2007	76	Immunohistochemistry	Cetuximab	$P < 0.0007$	Yes (resistance)	–	[82]
FCGR2A-H131R	Zhang	2007	39	Amplification and sequencing	Cetuximab	$P = 0.14$		–	[84]
FCGR3A-V158F polymorphism						$P = 0.057$			
						$P = 0.003$ (both)			
Circulating VEGF	Vincenzi	2007	45	Enzyme-linked immunosorbent assay	Cetuximab	$P < 0.014$	Yes (response)	–	[85]
PIK3CA mutation + PTEN expression	Jhaver	2008	<i>In vitro</i>	Direct sequencing western blot	Cetuximab	$P = 0.008$	Yes (resistance)	KRAS mutations, BRAF mutations	[81]

value; the predictive significance is not completely absolute, in fact a small subgroup of K-ras mutant patients respond to cetuximab and not all K-ras wild-type patients respond, demonstrating that only K-ras mutational status or a single factor does not completely discriminate responder from nonresponder patients, due to alternative factors involved in the downstream EGFr signalling pathway; the EGFr status, such as the semiquantitative expression detected by IHC, or quantitative measure of protein level, or the gene copy number, has shown different results in relation to the different assays used in the studies. However, this aspect is important especially in relation to the development of the PET probes in clinical setting that are aimed to detect EGFr expression in tumours. In fact, the EGFr expression is not predictive probably for methodical problems but it may also be due to the fact that the receptor total amount is not determinant in CRC biological background. On the contrary, the activation status measured as phosphorylated receptor on tyrosine residue 1068 may be more implicated in tumour progression and tumour maintenance. In this case, EGFr activity should be considered a better target than EGFr content for *in vivo* molecular imaging studies. Therefore, labelled EGFr TK inhibitors could also be suitable in CRC or labelled monoclonal antibodies recognising specifically the phosphorylated receptor should be investigated as a candidate PET biomarker for EGFr expressing colorectal tumours.

To conclude the clinical scenario, the large amount of molecular data accumulated over the last years in NSCLC and CRC dramatically improved the knowledge of EGFr role in solid tumours. However, the treatment prediction is still an open question in clinical practice. The *in vivo* detection of EGFr with molecular imaging technologies may provide some advantages that overcome several limits related to the tissue specimen analysis [86]. First of all, the information which comes from these analyses was derived from small tissue specimens and may not be representative of whole tumours. Secondly, most of the discussed studies have been conducted on primary tumours. Differences between primary tumours and its metastases as well as differences between metastases themselves have been reported. Thirdly, biological changes during the natural history of metastatic disease especially in long survival or heavily pre-treated patients may occur. It is possible that acquired mutations of K-ras or EGFr occur during the metastatic process explaining why not all wild-type K-ras CRC patients or EGFr-mutated NSCLC patients benefit from anti-EGFr-based therapy. In addition, validated molecular methods for the assessment of tumour biological background for routine use are not available, because the previous studies were not often comparable on the basis of the method used. Finally, invasive, nonrepeatable and time-consuming procedures for both patients and physicians are required. As a consequence, the *in vivo*, global, noninvasive detection of the distribution or of the activity of EGFr in metastatic disease may represent a big and potential tool for many oncologists in clinical practice.

PET probes for *in vivo* EGFr detection

The development of PET bioprobes as a tool for molecular imaging of EGFr seems to be one of the most interesting topics of cancer research. Two major strategies for visualising EGFr

have been studied: labelling small molecules such as TK inhibitors and labelling monoclonal antibodies.

labelling TK inhibitors

Until now, many labelled reversible and irreversible TK compounds were synthesized, each one with good chemical properties and high inhibitory potency *in vitro* [12, 87] (Table 3).

One of the first labelled compounds, ^{11}C -4-(3-bromoanilino)-6,7-dimethoxyquinazoline (^{11}C -PD153035), was reported in 1999 by Fredriksson et al. [88] in human neuroblastoma xenograft rats and obtained some really preliminary results on the *in vivo* biodistribution of the tracer. In 2001, Bonasera et al. [89] synthesized five 4-(anilino)quinazoline derivatives (ML01) radiolabelled with fluorine-18 (^{18}F) in the aniline moiety. These compounds are structurally analogue to the prototype small molecules PD153035 and bind reversibly to the ATP-binding domain of EGFr, blocking the autophosphorylation of the receptor. The *in vitro* experiments carried out in A431 human epidermoid carcinoma cell line showed that ML01 are potent inhibitors of EGFr autophosphorylation and of cell growth. However, the *in vivo* biodistribution experiments with PET, carried out in tumour-bearing nude mice, demonstrated a high non-tumour-specific uptake of ^{18}F -ML01, due to the rapid washout of the tracer, probably explained by a high competition at the compound's binding site with intracellular ATP.

In order to achieve better results *in vivo*, the same research group developed the radiosynthesis of N-{4-[(4,5-dichloro-2-fluorophenyl)amino]quinazolin-6-yl}-acrylamide (ML03), an irreversible EGFr TK inhibitor, that binds covalently to the cys-773 proximal to the ATP-binding site [90, 91]. This molecule was labelled with carbon-11 (^{11}C) at the acryloyl amide group. In comparison to the previous reversible compound, the ^{11}C -ML03 showed higher affinity for EGFr, higher inhibitory potency and an irreversible binding effect in the same cell line. Even if the formation of covalent binding solved the problem of the fast washout of the tracer, the *ex vivo* biodistribution studies in A431 tumour-bearing rats showed a low accumulation of ML03 in the tumour in comparison to the liver, kidney and intestine accumulation and the *in vivo* PET studies showed a weak tumour uptake. The authors postulated and concluded that the chemical properties, the rapid metabolism and the short life make this tracer unsuitable for EGFr molecular imaging studies.

The attempt to overcome the previous difficulties has led to the development of a new generation of irreversible EGFr TK inhibitors, characterised by a more chemical and biological stability. Shaul et al. [92] replaced the acrylamide group of ML03 with other more potent functional groups (α -methoxy-acetamide, α -chloro-acetamide, 4-dimethylamino-but-2-enoic amide) and radiolabelled these new inhibitors with the longer-lived radionuclide, iodine-124 (^{124}I). Only *in vitro* studies were carried out on A431 cells. The inhibitory potency was found to be higher than ML03. The longer half-life of the ^{124}I in comparison to ^{11}C seemed promising for prolonging the range of time for imaging after injection of the radiotracer. Mishani et al. [93] also reported a new generation of TK inhibitors, 4-dimethylamino-but-2-enoic acid [4-(phenylamino)-quinazoline-6-yl]-amides (ML04) that

Table 3. Positron emission tomography probes developed for epidermal growth factor receptor (EGFr) imaging in cancer

Authors	Years	Molecules	Compound	Chemical properties	Radiolabeling	<i>In vivo/in vitro</i> studies	Cancer cell lines	Ref.
Fredriksson	1999	Tyrosine kinase inhibitor	4-(3-bromoanilino)-6,7-dimethoxyquinazoline	Reversible	¹¹ C	<i>In vivo</i>	SH-SY5Y	[88]
Bonasera	2001	Tyrosine kinase inhibitor	4(anilino)quinazoline derivatives (ML01)	Reversible	¹⁸ F	Both	A431	[89]
Ortu	2002	Tyrosine kinase inhibitor	N-4-[(4,5-dichloro-2-fluorophenyl)amino]quinazolin-6-yl-acrylamide (ML03)	Irreversible	¹¹ C	Both	A431	[91]
Ben-David	2003	Tyrosine kinase inhibitor	Acryloyl chloride	Irreversible	¹¹ C	–	–	[90]
Shaul	2004	Tyrosine kinase inhibitor	A-methoxy-acetamide (4), α -chloro-acetamide (5), 4-dimethyl-amino-but-2-enoic amide	Irreversible	¹²⁴ I	<i>In vitro</i>	A431	[92]
Mishani	2004	Tyrosine kinase inhibitor	4-dimethylamino-but-2-enoic acid [4-(phenylamino)-quinazoline-6-yl]-amides (ML04)	Irreversible	¹¹ C	<i>In vitro</i>	A431	[93]
Mishani	2005	Tyrosine kinase inhibitor	4-dimethylamino-but-2-enoic acid [4-(phenylamino)-quinazoline-6-yl]- amide group (ML05)	Irreversible	¹¹ C	Both	A431	[94]
Pal	2006	Tyrosine kinase inhibitor	(E)-But-2-enedioic acid [4-(3-[¹³¹ I]iodoanilino)-quinazolin-6-yl]-amide-(3-morpholin-4-yl-prpyl)-amide (morpholino-IPQA)	Irreversible	¹³¹ I	Both	A431, U87MG, U87 Δ EGFr	[55]
Wang	2006	Tyrosine kinase inhibitor	Iressa (gefitinib)	Reversible	¹¹ C	–	–	[100]
Wang	2007	Tyrosine kinase inhibitor	4-N-(3-bromoanilino)-6,7-dimethoxyquinazoline (PD 153035)	Reversible	¹¹ C	Both	MDA-MB-468, A549, MDA-MB-231	[98]
Wang	2007	Tyrosine kinase inhibitor	4-N-(3-bromoanilino)-6,7-dimethoxyquinazoline (PD 153035)	Reversible	¹¹ C	Both	MDA-MB-468, A549, MDA-MB-231	[97]
Su	2008	Tyrosine kinase inhibitor	Gefitinib	Reversible	¹⁸ F	Both	U87, U87-EGFr	[99]
Abourbeh	2007	Tyrosine kinase inhibitor	4-dimethylamino-but-2-enoic acid [4-(phenylamino)-quinazoline-6-yl]-amides (ML04)	Irreversible	¹⁸ F	Both	A431, PC10, MDA-MB-468, NCI-H1975	[95]
Dissoki	2007	Tyrosine kinase inhibitor	PEG-ML04/ML05	Irreversible	¹⁸ F	<i>In vitro</i>	A431	[96]
Perk	2005	mABs	cetuximab	Reversible	⁸⁹ Zr, ⁹⁰ Y ¹⁷⁷ Lu	Both	A431	[101]
Cai	2007	mABs	DOTA-cetuximab	Reversible	⁶⁴ Cu	Both	U87MG PC3, CT-26 HCT-8, HCT-116, SW620, MDA-MB-435	[102]
Ping Li	2008	mABs	DOTA-cetuximab	Reversible	⁶⁴ Cu	Both	A431, MDA, MB-435	[103]
Velikyan	2005	mABs	DOTA-hEGF	Reversible	⁶⁸ Ga	Both	U343, A431	[104]

were radiolabelled with ^{11}C . Similarly, the *in vitro* results carried out showed the high specific activity and the high chemical purity of ML04 that may be translated in a higher biological stability, in a lower degradation rate and then in a higher specific uptake in EGFr-positive tumours. These compounds had achieved promising results in the *in vitro* experiments, but the efficacy was not evaluated *in vivo*. In a following study, ^{11}C -ML04 was evaluated both *in vitro* and *in vivo* and was also compared with ^{11}C -ML03 [94]. The stability of ^{11}C -ML04 was higher than ^{11}C -ML03 in fact the extractable intact tracer from blood 15 min post-injection into nude rats was 50% higher and the total extractable intact tracer within 60 min following injection was three-fold higher. However, even though these results were encouraging and even though the subsequent *in vitro* experiments carried out on A431 cells, MDA-MB-468 breast carcinoma, PC10 lung carcinoma and NCI-H1975 NSCLC cells confirmed the selective irreversible receptor inhibition of ML04, the *in vivo* distribution studies using ^{18}F -ML04 in tumour-bearing U87MGwtEGFr and U130MG nude mice were quite disappointing because showed lower uptake in U87MGwtEGFr tumour activity in comparison to other organs. These data were confirmed in a control study evaluating the significant levels of activity uptake in control EGFr-negative U138MG tumours and the blocking studies resulted in higher blood activity concentration in target tissues and in high blood activity levels of the nonlabelled compound [95].

Another research group, Pal et al. [55], developed the synthesis of ^{124}I radiolabelling of (E)-But-2-enedioic acid [4-(3-[^{124}I]iodoanilino)-quinazolin-6-yl)-amide-(3-morpholin-4-yl-propyl)-amide (morpholino-[^{124}I]-IPQA), which selectively and irreversibly binds the ATP-binding site specifically of the 'phosphorylated' EGFr TK. The *in vitro* studies were carried out in three different cell lines (A431, U87MGwtEGFr and U87ΔEGFr expressing the EGFr-vIII mutant cell lines) and showed a progressive retention of morpholino-[^{124}I]-IPQA in A431 cells and in U87ΔEGFr human glioma cells which have the receptor highly activated, but not in U87 wild-type cells with low kinase activity. The *in vivo* studies were carried out using small animal PET in bearing A431 cells and K562 xenograft rats and in A431 cells and K562 xenograft mice. They demonstrated a significant difference in the accumulation of the radiotracer in A431

carcinoma tumour xenografts that reflects the EGFr expression and EGFr activity in comparison to K562 tumour control xenografts (Figure 1). However, the morpholino-[^{124}I]-IPQA exhibited a rapid hepatobiliary clearance especially in rats that limits the possibility of using this tracer for imaging liver metastases. Moreover, PET imaging was not carried out in U87ΔEGFr human glioma xenograft mice. The authors concluded that morpholino-[^{124}I]-IPQA may be a potential tool for the identification of high EGFr kinase activity in tumours, especially brain tumours expressing EGFr-vIII mutants and NSCLC expressing activating mutations in the EGFr kinase domain.

The last and more recent groups of irreversible compounds are the novel pegylated ^{18}F inhibitors on the basis of the same 4-anilinoquinazoline structure as in ML04 and ML05, attaching at the 7-position of the quinazoline ring various lengths of polyethyleneglycol (PEG) chains [96]. This novel chemical modification was carried out in order to increase stability and solubility of tracers. At the moment, *in vivo* studies of these new compounds are ongoing. The *in vitro* experiments in A431 cells indicated that pegylated derivatives of ML04 have higher solubility and lower lipophilicity in comparison to the parent compounds, associated with a high irreversible potency of EGFr autophosphorylation inhibition. They seem very promising as agent for molecular imaging of EGFr.

Different papers on the labelling of reversible compounds (analogous to PD153035 and ML01) were also recently published with disappointing results [97–99]. Gefitinib, which is a reversible EGFr TK inhibitor, was labelled with ^{11}C or ^{18}F [99, 100]. Unfortunately, the *in vivo* PET/CT imaging studies of ^{18}F -gefitinib showed no uptake in EGFr expressing tumours for rapid hepatobiliary clearance. The lack of EGFr-specific tumour uptake of labelled ^{18}F gefitinib makes it unsuitable as PET probe for noninvasive assessment of EGFr status in patients, but useful for the assessment of gefitinib's pharmacokinetics by PET imaging.

The failure of anti-EGFr labelled tracers may indicate that the selection of patients who are likely to benefit from EGFr small molecules inhibitors could not be on the basis of EGFr tumour levels.

Over the last few years, the chemical properties of TK inhibitors compounds as potential PET probes have been improved thanks to the modification of their molecular

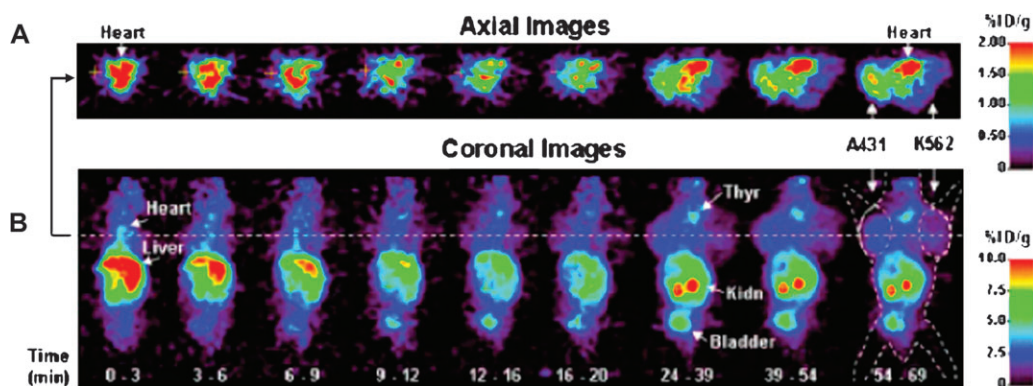


Figure 1. Small animal positron emission tomography images with morpholino- ^{124}I -IPQA in A431 and K562 xenograft tumour model. Axial images (A) radiotracer uptake in A431 and K562 tumours is indicated by white arrows. Coronal images (B) radiotracer routes clearance (reprinted from [55]).

structure. However, until now, none of them have shown the biological qualities to be used for molecular imaging of EGFr-positive tumours. Further studies and more efforts are required in order to find a clinical translation.

labelling monoclonal antibodies

Labelling monoclonal antibodies with PET radioisotopes represents another strategy for the *in vivo* visualisation of EGFr tumour expression but it seems more complicated. In comparison to labelling TK inhibitors, few studies have been conducted with the attempt of synthesizing monoclonal antibody-based probes [101–103]. Cetuximab is the first monoclonal antibody targeting EGFr approved in clinical setting for treatment of patients with CRC and head–neck cancer and the first one labelled with PET radioisotopes. In 2005, ⁸⁹Zr-labelled cetuximab was synthesized and used not for imaging purpose but for evaluating *in vivo* biodistribution of therapeutical labelled radiometals ⁹⁰Y and ¹⁷⁷Lu because cetuximab is able to internalise after binding with the receptor [101]. DOTA-cetuximab labelled with ⁶⁴Cu (⁶⁴Cu-DOTA-cetuximab) was first reported by Cai et al. [102] in 2007 as a potential PET probe. The authors synthesized this tracer and investigated its EGFr imaging potential both in seven tumour cell lines and in seven xenograft tumour models (Figure 2). PET studies demonstrated that the tumour uptake of ⁶⁴Cu-DOTA-cetuximab was significantly higher in U87GM and PC3 tumour models that are characterized by higher EGFr expression in comparison to other ones. Moreover, they

found a close correlation between the tumour uptake and the tumour EGFr expression level measured by western blotting assay. Ping et al. [103] reported the results of the same PET probe showing a higher tumour uptake in EGFr-positive A431 tumour models in comparison to EGFr-negative MDA-MB-435 tumour model. Both studies reported that ⁶⁴Cu-DOTA-cetuximab has a relatively high molecular weight and it is excreted through the hepatic route and it could, therefore, probably give limited information about liver tumours. ⁶⁸GA-DOTA-hEGF, a tracer with labelled EGF, the natural ligand of EGFr, was also developed [104].

On the contrary to TK inhibitor-based probes studies, the authors concluded that ⁶⁴Cu-DOTA-cetuximab is a promising agent for *in vivo* detection of EGFr and could be translated in the clinical setting. Some considerations on these conclusions will be reported.

critical points of *in vivo* application of anti-EGFr PET probes

Until now, none of some above discussed PET tracers were evaluated in patients. Despite the theoretical rationale of EGFr imaging is strong, despite the *in vitro* and *in vivo* results seem promising and the EGFr imaging seems feasible, the translation of this approach in the clinical setting needs to be considered with caution for several reasons. Critical points or obstacles to their clinical development need to be briefly considered, in particular some technical aspects that may limit the high-quality images in patients and also some aspects on the legislative process that may preclude their easy study in humans.

in vivo imaging key problems

First of all, it may be difficult to exactly determine the *in vivo* specificity target binding of the tracer, even the *in vitro* results showed a high potency in EGFr binding or inhibition especially for TK inhibitors compounds. For this reason, the strict correlation between the tumour specific uptake and the receptor expression/deregulation/activation is not always well demonstrated for all investigated tracers [91, 95]. From a clinical point of view, the definition of ‘EGFr-positive tumours’ is possible only in the case of positive images even though we also have to consider the possibility that the tracers may bind other receptors with similar affinity to EGFr. In case of negative images, we are not able to define the tumour as ‘EGFr negative’. The non-EGFr-specific tumour accumulation observed in preclinical studies especially for TK inhibitor compounds was explained by several factors. First, tumour characteristics such as vascularisation or necrosis may interfere with the tracers’ uptake. Secondly, the rapid hepatobiliary excretion of the tracers may reduce their bioavailability [95, 99]. The biodistribution studies showed a high activity in nontarget organs such as the liver, intestine or kidney [55, 91, 95, 99]. The lack of EGFr-specific tumour uptake led some authors to conclude that TK inhibitor-based tracers are unsuitable for *in vivo* EGFr imaging. On the contrary, some authors in order to enhance the tumour-specific uptake indicate to modifying the route of administration such as prolonged intravenous infusion or modify the chemical properties [87, 95]. In fact, the ¹⁸F-PEG anilinoquinazoline

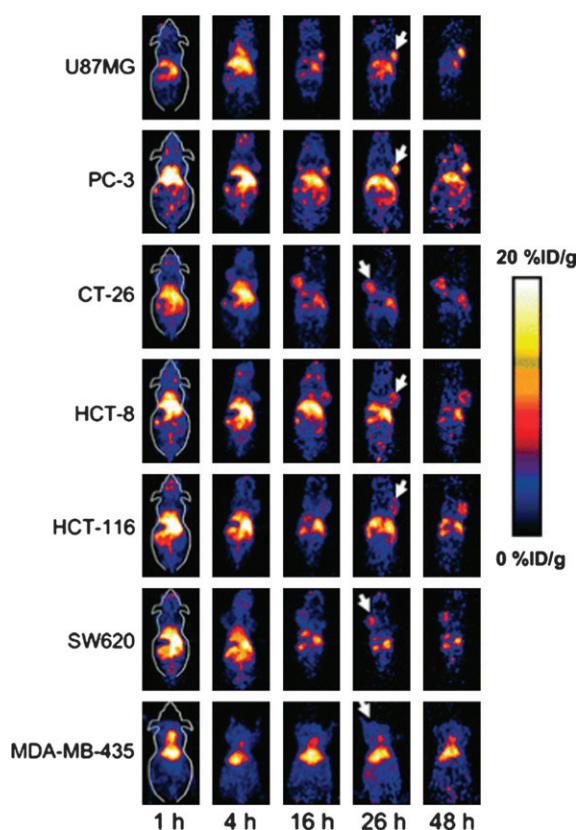


Figure 2. Small animal positron emission tomography images with ⁶⁴Cu-DOTA-cetuximab in seven xenograft tumour models. Radiotracer uptake in tumours is indicated by white arrows (reprinted from [102]).

derivatives were developed for increasing the lipophilicity and decreasing the solubility [96]. These agents seem really promising and the imaging studies are still going on. However, we have to consider and suppose that in patients, the nonspecific uptake could be due also to EGFr itself because the receptor status may not be well known and uniform as in animal models.

A second critical point is that some tracers especially the mAb-based ones have a long half-life that may limit the possibility of the acquisition of serial images in humans. Moreover, they have a relatively high molecular weight like ^{64}Cu -DOTA-cetuximab and are also excreted through the hepatic route limiting the application on imaging of liver tumours. However, >70% of patients with CRC develop liver metastases and a novel PET tracer able to give information on liver status could be really important for these patients because only a combined approach such as chemotherapy, surgery, locoregional treatments of liver metastases improves the survival [105]. Smith-Jones et al. [106, 107], who first conjugated the DOTA-trastuzumab with ^{111}In and ^{64}Cu for imaging of HER-2-expressing tumours in breast cancer BT-474 xenografts expressing high levels of this receptor, try to conjugate the minibody of trastuzumab to ^{68}Ga in order to shorten the half-life. More recently, Cheng et al. [108] also developed ^{18}F -labelled anti-HER2 Affybody molecules (^{18}F -FBO- $Z_{\text{HER2:477}}$) and evaluated this new tracer in SKOV3 cell lines with high HER2 expression levels and in xenograft mice. This approach using the antibody-derived fragments should be evaluated also for anti-EGFr monoclonal antibodies in order to facilitate their clinical application.

Considering all the key problems above, the ideal PET probe for clinical purposes would have several specific characteristics related to the target molecules, to the compound and to labelling (Table 4). Moreover, in order to candidate a compound as PET probe, it is mandatory to confirm all the adequate characteristics in a correct step sequence of experiments. The *in vitro* studies should be carried out in cell lines which are highly, moderately and also not expressing the target as a control in order to define the target-binding specificity. The correct *in vivo* animal model should first be well established by having the molecular characteristics of interest (target expression or target activity) evaluated by *ex vivo* studies in order to define the presence of the target. Secondly, the

Table 4. Main characteristics of the ideal positron emission tomography probe

Target	Well known Highly expressed in tumours Differentially expressed in comparison to the nonpathological tissues
Tracer	Good target binding affinity Good target binding selectivity Ideal chemical properties (lipophilicity or solubility depending on the cellular site) Stability Slow washout from the tumour Not long half-life limiting serial images in human studies Low hepatic excretion in order to visualise liver metastases
Labelling	Adequate half-life for human studies

imaging studies should be carried out by using the appropriate animal model and then compared with animal control models which do not have the target of interest and with animal control models which do not have tumours. The tracers imaging may also be compared with other standard PET probes such as fluorodeoxy-glucose or fluoro-3-deoxy-thymidine. The mass should be small avoiding the presence of necrosis. Finally for a correct interpretation of the images, morphological and molecular analyses are necessary after animal sacrifice in order to evaluate necrosis, vascular characteristics and obviously the target expression or activation.

clinical development process of a new PET probe

In vivo molecular imaging represents one of the most interesting approaches as a potential tool for predicting the efficacy of targeted therapies. PET tracers for EGFr and the large amount of new ones are synthesized for clinical application purposes. However, we have to underline that the clinical development of new PET tracers is very difficult [109]. The regulatory processes for the approval of the molecular imaging probes are different in every country in the world. In general, it is necessary to produce a pharmacology and toxicology dossier, to provide all the information on the chemical manufacturing properties and to design an appropriate clinical study. However, everywhere these processes are too long and too expensive and require a lot of effort especially for academic researchers. The guidelines for 'first in humans pharmaceuticals development' provided by European Medicines Agency and the Food and Drug Administration require the following steps: from the *in vitro* studies to small animal studies, to nonclinical safety studies for the final application to patients. The products obviously should be prepared in good medical practice conditions which may represent one of the most important limitations for many academic centres. As a consequence, despite the large amount of preclinical studies in molecular imaging and despite the preclinical promising results, most novel PET probes will be never translated in the clinical setting. However, considering the clinical urgency to incorporate molecular imaging technologies in practice, the regulatory processes need to be revised around the world in order to accelerate the process and to decrease the costs. Moreover, specific expert institutions for approving the imaging probes are needed. Some local committees or local government units have been specifically created for the development of molecular imaging probes such as the local Radioactive Drug Research Committee (RDRC) in United States.

Finally, as a last consideration, cyclotron and PET technologies are not available everywhere and also in some countries the costs may not facilitate PET studies in everyday practice.

conclusions

The knowledge of biological abnormalities of the solid tumours seems to be a crucial point in which targeted agents can be applied to cancer care. EGFr is considered one of the most studied molecules as target for cancer therapy. Over these last few years, several studies attempting to identify predictive biomarkers of treatment response, such as the receptor status or other molecules related to the downstream signalling pathway, have been conducted. However, from a clinical point of view,

the information obtained from *ex vivo* analyses still have various limitations that may be overcome by the combination with a noninvasive, global and *in vivo* evaluation of the molecular tumour background.

Two classes of PET probes have been developed for *in vivo* detection of EGFR in cancer: labelled small molecules such as TK inhibitors and labelled monoclonal antibodies. Until now, they have been evaluated only in preclinical studies. The *in vitro* and *in vivo* results of all PET tracers are very different depending on the chemical properties, positron emission radionuclide, or animal models. As a consequence, various critical questions are still open and their potency to be translated in the clinical setting for EGFR imaging in cancer patients requires further efforts. On the agenda for future research are first of all the need to perform imaging studies with the PEG-TK inhibitor compounds; secondly, novel compounds with chemical properties for recognising the activated or phosphorylated status of the receptor should be synthesized and thirdly the antibody-derived fragments should be further investigated.

In conclusion, imaging technologies like PET may be useful to *in vivo* evaluate the distribution or the activation of EGFR in cancer and represent a big challenge for future cancer research perspectives. However, the optimism derived from some preclinical studies and the enthusiasm of physicians to have a potential tool for predicting and monitoring anti-EGFR treatments in clinical practice still need some caution.

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