

Recent Results in Cancer Research  
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Michael Baumann  
Mechthild Krause  
Nils Cordes *Editors*

# Molecular Radio- Oncology

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# Recent Results in Cancer Research

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# Molecular Radio-Oncology

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## Preface

This textbook on *Molecular Radio-Oncology* is targeting physicians and preclinical researchers with interest in translational radiation oncology.

The first part of the book takes up current knowledge about important molecular radiobiological mechanisms and preclinical investigations on target identification and personalization for combined treatments, DNA repair, and cancer stem cells.

In the second part, potential biomarkers for personalized radiation oncology are described from their preclinical basis to translational and clinical data. The epidermal growth factor receptor (EGFR) and tumor hypoxia are examples of long-identified biomarkers that over time have been used as both prognostic markers and targets for combined treatments. Human papillomavirus infections are increasingly evident in different tumor entities, have been shown to determine prognosis of the patients, and are currently tested as basis for interventions in clinical trials.

The third part of the book refers to the utilization of radiobiological knowledge for the application of molecular imaging techniques in radiation oncology. Fluoro-deoxyglucose (FDG) is the most widely used tracer for positron emission tomography (PET) and indicates not only active tumor tissue with an often higher sensitivity compared to standard sectional imaging techniques, but has also prognostic value for the outcome of radiotherapy. <sup>18</sup>F-Misonidazole PET is described as an example of bioimaging of a factor of radioresistance—under the view of utilization as a biomarker for the outcome of radiotherapy as well as of automatic contouring methods for reproducible evaluation.

All chapters are authored by eminent researchers and physicians in the respective fields with a broad experience in preclinical and clinical radiation oncology and long-term teaching experiences who have put a focus on clarity and comprehensiveness of the content of the different chapters.

Michael Baumann  
Mechthild Krause  
Nils Cordes

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All three editors are in addition associated with the Helmholtz-Zentrum Dresden-Rossendorf, where radiopharmacy, physics, biology, and clinical research are jointly performed for the improvement of radiooncological patient treatment.



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# DNA Repair

Kerstin Borgmann, Sabrina Köcher, Malte Kriegs,  
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## Abstract

Cellular chromosomal DNA is the principal target through which ionising radiation exerts its diverse biological effects. This chapter summarises the relevant DNA damage signalling and repair pathways used by normal and tumour cells in response to irradiation. Strategies for tumour radiosensitisation are reviewed which exploit tumour-specific DNA repair deficiencies or signalling pathway additions, with a special focus on growth factor signalling, PARP, cancer stem cells, cell cycle checkpoints and DNA replication. This chapter concludes with a discussion of DNA repair-related candidate biomarkers of tumour response which are of crucial importance for implementing precision medicine in radiation oncology.

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## Keyword

Ionising radiation · DNA damage response · DNA strand breaks · DNA double-strand break repair · Molecular targeting · Biomarkers

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## 1 Introduction

DNA is a vitally important biomolecule which stores the genetic information required to create the molecular building blocks for cells, tissues and whole organisms. At the same time, it is surprisingly prone to damage and decay, even in the absence of any exogenous stressors. To counteract endogenous processes such as oxidation and hydrolysis as well as exogenously induced lesions, cells have devised a range of pathways for repairing damaged DNA, without which higher life forms would not have been able to evolve.

Not surprisingly, cellular DNA is also the principal target through which ionising radiation exerts its main biological effects, whether cell killing, neoplastic transformation, mutation induction, growth arrest or cellular ageing (UNSCEAR 2000). Whilst the main aim of radiotherapy is tumour cell inactivation, one needs to consider all of these biological responses to get the full picture of how radiotherapy affects the tumour and the surrounding normal tissue. And in order to understand these cellular responses, it is important to know about the molecular machinery that cells employ to repair DNA that has been damaged by radiation.

Apart from furthering our understanding of the basic mechanisms that govern the cellular radiation response, research into DNA repair also opens up opportunities to (i) learn why some individuals may react more severely to radiotherapy than others, (ii) identify potential markers of individual tumour and patient response to support a move towards personalised treatment and (iii) establish biological targets that can be used for tumour radiosensitisation.

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## 2 Radiation-Induced DNA Damage and Early Cellular Responses

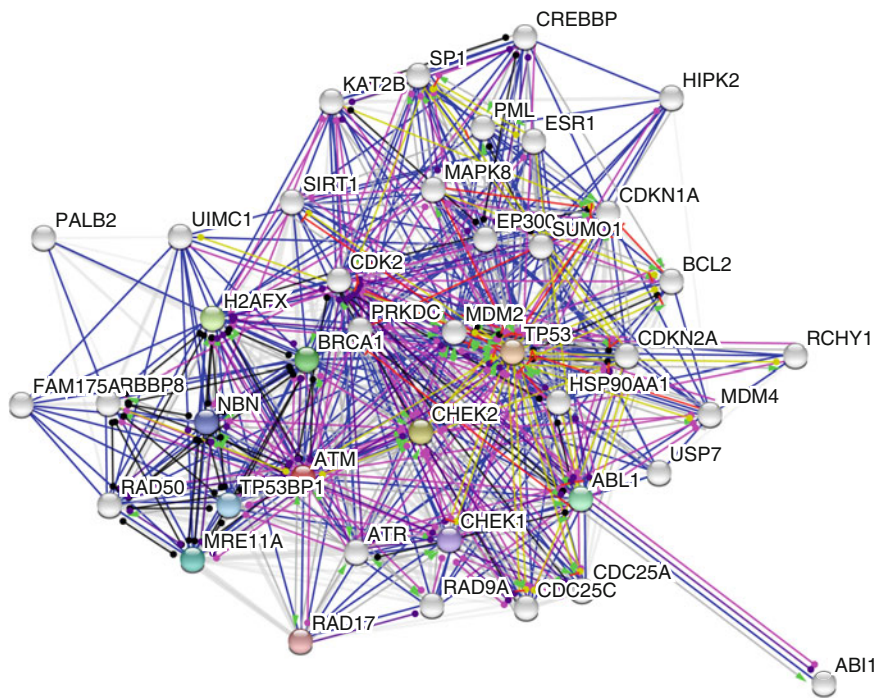
Radiation can damage chromosomal DNA either directly or indirectly via the production of free radical species (such as the hydroxyl radical) in the immediate vicinity of the DNA. The breakage of chemical bonds in the sugar–phosphate backbone of the DNA may result in the formation of strand breaks. Other types of lesions induced by ionising radiation include altered base and sugar moieties as well as cross-links which may form between proteins and DNA. All these modifications are not randomly distributed across the cell nucleus; instead, they form along the track of each ionising particle, as it deposits its energy. The resulting clustered lesions consist of multiple DNA lesions which are closely spaced within a volume of several nanometres, corresponding to up to about 20 base pairs. Occasionally, two strand breaks may be induced on opposite strands within one clustered lesion. These may cause the DNA molecule to break up, if there is insufficient overlap to maintain the DNA double helix via the weak hydrogen bonds between the paired, complementary bases. The resulting DNA double-strand breaks (DSBs) are very

deleterious. If left unrepaired, they may cause DNA degradation and loss of chromosomal fragments during the next mitosis. Furthermore, their faithful repair is complicated by the fact that, in contrast to lesions that affect only one of the two DNA strands, there is no template immediately available that could be used to correctly restore the original DNA sequence. For this reason, DSB ends are prone to be misrepaired, causing either small-scale mutations at the break point or chromosomal rearrangements such as translocations if break ends from multiple breaks interact and get misrejoined (Rothkamm and Lobrich 2002). It is for these reasons that DSBs are believed to be the most important DNA lesions induced by ionising radiation. For the same reasons, the remainder of this chapter will focus mainly on the cellular response to DSBs.

Cells respond to ionising irradiation through a highly interactive functional network of partially overlapping DNA damage response pathways (Sulli et al. 2012; Jackson and Bartek 2009). Upon detection of DSBs or extended stretches of single-stranded DNA, molecular DNA damage sensors such as the MRE11-RAD50-Nibrin (MRN) and the KU70-KU86 complexes or RPA, ATRIP and the RAD9-RAD1-HUS1 complex will activate the apical kinases ATM, DNA-PK or ATR, respectively, which belong to the family of phosphatidylinositol 3' kinase-related kinases. These will in turn phosphorylate a plethora of DNA damage mediators and downstream kinases. Examples of mediators—which frequently accumulate at or in the vicinity of DSBs to form microscopically visible DNA damage foci—include MDC1, 53BP1, H2AX, BRCA1 and TOPBP1. Downstream kinases include the checkpoint factors CHK1 and CHK2. Figure 1 exemplifies some of the known and predicted protein interactions for the key DNA damage kinase ATM, according to STRING 10 database (Jensen et al. 2009).

The main outcomes of the DNA damage response include the following:

- (i) Transcriptional activation or repression of DNA damage-responsive genes.
- (ii) Restriction of cell cycle progression at DNA damage-induced checkpoints in order to allow DNA repair to proceed before the cell enters S phase or mitosis. CDC25 and p53 are important effectors to facilitate this outcome.
- (iii) Post-translational modification (phosphorylation, acetylation, ubiquitylation, sumoylation, neddylation) of chromatin constituents and associated proteins around the site of the DNA lesion to facilitate repair (Bekker-Jensen and Mailand 2011; Brown and Jackson 2015).
- (iv) Repair of DNA lesions, possibly resulting in mutations and chromosomal aberrations.
- (v) Induction of apoptosis in radiation-damaged cells, which may occur via p53 or in a p53-independent manner.
- (vi) Induction of autophagy, probably via inhibition of mammalian target of rapamycin complex 1 (mTORC1) (Czarny et al. 2015).



**Fig. 1** Network of some of the proteins that interact physically or functionally with ATM, according to STRING 10 database (<http://string-db.org>; accessed 4 November 2015). *Line colours* indicate the nature of the physical or functional interaction: *green*—activation; *red*—inhibition; *blue*—binding; *turquoise*—phenotype; *purple*—catalysis; *pink*—post-translational modification; *black*—reaction; *yellow*—expression

### 3 Repair of Single-Stranded DNA Lesions

Several mechanisms for repairing ionising radiation-induced DNA damage exist in human cells which have some overlapping functions and may act as backup pathways for each other to minimise the risk of any damage being left unrepaired. Single-strand breaks and damaged bases such as 7,8-dihydro-8-oxoguanine or thymine glycol are the most common lesions induced by ionising radiation and also form very frequently endogenously, thus requiring efficient repair mechanisms to maintain genome integrity. Thanks to the availability of the complementary sequence on the intact opposite strand which serves as a template for repair, these lesions do not normally give rise to any mutations.

Single-strand breaks caused by damage to the sugar moieties in the DNA are detected by and activate poly(ADP-ribose)polymerase 1 (PARP1) which subsequently modifies itself and other proteins with chains of hundreds of ADP-ribose units. These recruit XRCC1 protein complexes (probably containing DNA polymerase beta,

ligase 3 and one of PNKP, APTX or APLF) which process DNA ends. Two alternative options exist for the final two steps, gap filling and DNA ligation, namely short-patch and long-patch repair during which either one or 2–10 nucleotides are incorporated by polymerase beta and/or polymerase delta/epsilon, resulting in the displacement of the damaged strand in the latter case which is then removed by FEN1/PCNA, possibly also with support from PARP1. Repair patches are sealed by the XRCC1/ligase III alpha or PCNA/ligase I complexes (Caldecott 2014).

Base lesions are essentially repaired by the same base excision repair (BER) process described above for single-strand break repair, except that this pathway initially employs lesion-specific glycosylases to remove altered bases and then converts the abasic sites into single-strand breaks via an AP endonuclease activity (Brennerman et al. 2014). These breaks are then processed in the same way as those directly induced by radiation.

In global nucleotide excision repair, bulky lesions which distort the helical DNA structure can be recognised by the XPC-hHR23B-centrin 2 complex which then melts the DNA around the lesion and recruits the TFIIH complex. Subsequently, XPB and XPD unwind the DNA to form a bubble of about 30 nucleotides. XPA then binds the DNA near the 5' end of the bubble, and RPA binds the single-stranded DNA opposite the lesion to protect it from degradation. The first incision is then made by ERCC1-XPF and repair synthesis commences, displacing the damaged strand. Finally, XPG makes the second incision to remove the oligonucleotide contained the lesion, and the newly synthesised DNA patch is sealed by ligase I or ligase III alpha-XRCC1 (Spivak 2015).

In contrast to the above process, which only detects bulky lesions, a transcription-coupled excision repair pathway can repair any DNA lesion located within an actively transcribed gene. In this case, it is the stalling of RNA polymerase II-mediated transcription which detects the lesion and triggers the recruitment of transcription-coupled repair factors. The polymerase is then backtracked or removed to make space for repair which proceeds as described above for global nucleotide excision repair (Spivak 2015).

DNA strand breaks in cellular DNA are commonly measured using alkaline single-cell gel electrophoresis, also called the comet assay (Ostling and Johanson 1984; Olive 2009). In brief, cells are embedded into low-melt agarose and spread onto microscopy slides, lysed, electrophoresed in alkaline running buffer, neutralised and stained with a fluorescent DNA dye, such as ethidium bromide or Sybr Gold, and imaged using a fluorescence microscope. Whilst cells with intact, unirradiated DNA will show round nuclei ('heads') without any DNA leaking out of the nucleus into a tail, cells containing strand breaks will show a comet-like tail of fragmented DNA which migrated out of the nucleus towards the anode during electrophoresis. The percentage of DNA in the tail and the tail length can be measured, and the tail moment be calculated by multiplying the two as a robust indicator of the amount of damage present in the sample. As ionising radiation induces about 30 times more single-strand breaks than DSBs, the initial signal

directly after irradiation will be dominated by single-strand breaks. However, the kinetics for repairing single- and DSBs differ considerably, with the former being repaired faster, so that residual damage remaining several hours after irradiation will be greatly enriched for DSBs. In that respect, any signal remaining after many hours in an alkaline comet assay will largely reflect the level of residual DSBs, at least in cells that are BER-competent.

One strategy to measure base lesions utilises glycosylases/AP endonucleases that convert specific base lesions into single-strand breaks (Collins 2014). To this end, lysed cells embedded in agarose slides are incubated with commercially available purified enzymes such as formamidopyrimidine DNA glycosylase or Nth (endonuclease III) to detect oxidised purines or pyrimidines, respectively, prior to electrophoresis. The additional tail moment measured on top of that observed in a parallel sample without glycosylase treatment is then a measure of the amount of base damage. Detailed protocols have been developed in recent years for the application of comet-based assays for various BER and NER substrates in different types of biological material (Azqueta et al. 2013).

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## 4 Pathways for Repairing DNA Double-Strand Breaks

DSBs can be repaired by non-homologous end-joining (NHEJ) processes as well as homology-dependent recombination pathways. Whilst excision repair pathways for single-stranded DNA damage generally use the intact complementary strand as a template, ensuring that repair is mostly error-free, DSB repair is generally more challenging and frequently error-prone. This is especially the case in situations when several DSB ends are sufficiently close to be misaligned and exchanged during repair, giving rise to chromosomal deletions or rearrangements. Promiscuous repair in the presence of multiple DSBs is also responsible for the quadratic increase of deleterious chromosome aberrations with gamma- or X-ray dose, despite a dose-linear increase of DSBs (Barnard et al. 2013). It is therefore important to appreciate that DSB repair efficiency is an important determinant of genome stability and radiosensitivity.

Gel electrophoretic studies of double-stranded DNA fragments following irradiation and immunofluorescence microscopy-based scoring of DNA damage-associated protein ‘foci’ forming at the sites of DSBs have shown that initial radiation-induced DSB yields do not in general vary very much between genomic loci, organs or individuals. However, some variation has been reported for different tumour cells which cannot be explained by other confounding factors such as DNA content but which may be associated with cellular radiosensitivity (El-Awady et al. 2003; Rube et al. 2008; Rothkamm et al. 2003; Cheng et al. 2015).

X- or gamma-ray-induced DSBs are repaired with biphasic exponential kinetics with half-lives of 5–30 min and several hours, respectively. Repair may reach a plateau of residual, unrepaired and potentially also misrepaired, DSBs (Rothkamm and Lobrich 2003; Dahm-Daphi and Dikomey 1996). Longer half-lives and more residual breaks have been observed in cells exposed to densely ionising particles.

Whilst the time course of DSB repair is generally comparable between tumour cells, lymphocytes or normal fibroblasts, more variability seems to exist between different tumour cell lines. These may reflect bigger differences in the efficiency of DSB processing in tumours, likely caused by deficiencies or upregulation of particular DNA damage response pathways.

Chromatin structure and cell cycle stage also affect both efficiency and kinetics of DSB repair. DSBs in heterochromatic DNA regions are much more difficult to access by repair factors, require additional chromatin relaxation steps mediated by KAP1 and tend to be repaired more slowly (Goodarzi et al. 2008). Repair pathway utilisation depends very much on cell cycle position as it is affected by the availability of a sister chromatid (Rothkamm et al. 2003; Bauerschmidt et al. 2010). Consequently, the radiation response of a normal tissue or a tumour may change during a course of fractionated radiotherapy if cells accumulate in a specific cell cycle phase. One example is the loss of fraction size sensitivity in the basal epidermal layer of irradiated skin which may be explained by the accumulation of cells in the S/G2 phase and consequential increase in the utilisation of homology-dependent repair mechanisms (Somaiah et al. 2012).

Repairing a DSB is of utmost importance for a cell because, if left unrepaired, it would lead to the loss of the affected chromosome fragment and cell death (Helleday et al. 2007). Furthermore, incorrect repair of a DSB may produce deletions or chromosome rearrangements which are hallmarks of cancer genomes. For these reasons, efficient DSB repair is of crucial importance for the survival and genomic stability of a cell. Human cells employ two fundamentally different types of DSB repair mechanisms, one relying on the joining of break ends with little or no regard for sequence homology, and the other heavily dependent on the availability of sequence homology. These will be discussed in more detail in the following sections.

## 4.1 End-Joining Mechanisms

The main DSB repair pathway in mammalian cells is classical non-homologous end-joining (NHEJ). It is active in all phases of the cell cycle (Lieber et al. 2003) except mitosis (Orthwein et al. 2014; Terasawa et al. 2014). NHEJ is initiated by the binding of the Ku70/Ku80 heterodimer to DSB ends, followed by recruitment and activation of the catalytic subunit of the DNA-dependent protein kinase to form the DNA damage kinase DNA-PK. Depending on the nature of the break, the ends may need to be trimmed before they can be ligated, e.g. by nucleolytic resection or by DNA polymerase-mediated fill-in. Then, the ligase complex consisting of ligase IV and its cofactors XRCC4 and XLF binds to the DNA ends and seals the break (Davis and Chen 2013). Intriguingly, whilst the basic NHEJ mechanism has been known for over twenty years, its regulation in human cells is still far from being fully understood.

Cells deficient in NHEJ can repair DSBs via an alternative end-joining mechanism (Alt-EJ) (Wang et al. 2003; Audebert et al. 2004) which is less efficient than



NHEJ and depends on PARP1 for DSB recognition and on ligase III and its cofactor XRCC1 for the ligation step (Mansour et al. 2010, 2013). It is associated with deletions of DNA at the break site, probably caused by the slower speed of the pathway which prevents nucleolytic degradation less efficiently than classical DNA-PK-dependent NHEJ (Mansour et al. 2013). Whilst Alt-EJ does not strictly require microhomology between overlapping bases at the break ends, it still preferentially uses it if available (Mansour et al. 2010). Interestingly, these features, i.e. deletions and microhomologies at breakpoint junctions, are frequently found in human cancer cells (Welzel et al. 2001; Weinstock et al. 2007; Jager et al. 2000).

## 4.2 Homology-Dependent Repair Pathways

The main homology-dependent repair pathway, homologous recombination (HR), operates at the replication fork as well as in post-replicative DNA, i.e. during S and G2 phase when the sister chromatid can serve as an intact template to facilitate repair of a DSB by restoring the original sequence. Repair via HR is therefore largely error-free. HR is initiated by a nucleolytic DNA end resection step during which single-stranded overhangs are generated. This is achieved by Mre11-CtIP, followed by Exo1, DNA2 and the BLM helicase (Helleday et al. 2007; Sung and Klein 2006). RPA protects the single-stranded DNA overhangs until RAD51 binds to form a nucleofilament, supported by the RAD51 paralogs RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 as well as by BRCA2 and RAD52. Following a search for DNA with extensive sequence homology, the RAD51 nucleofilament invades the double helix of, typically, the sister chromatid to form a so-called displacement or D loop. Using the complementary strand as a template, replication polymerases then synthesise DNA until ligase 1 seals the ends. The resulting branched structure of four double-stranded DNA arms joined together, called the Holliday junction, can be resolved either through symmetrical cleavage by GEN1/Yen1, through asymmetrical cleavage (Mus81/Eme1) or through dissolution via the BLM-TopIII-alpha complex to complete the repair process (Andersen et al. 2011; Sarbajna et al. 2014; Wyatt and West 2014).

A different homology-dependent repair mechanism for DSBs does not use a homologous copy such as the sister chromatid but instead utilises homologies between repetitive sequences on the broken chromosome. It is called single-strand annealing (SSA) and is also initiated by a nucleolytic end resection step to produce long single-stranded DNA overhangs to which RPA binds. Subsequently, heptameric rings of RAD52 form at the DNA overhangs and facilitate homology search and annealing of the repeat sequences. Next, the DNA sequences between the repeats are flapped out and cleaved off, most likely by the ERCC1/XPF endonuclease (Shinohara et al. 1998), and an as yet unknown ligase seals the remaining gap. It is clear from the above that SSA is a non-conservative process which results in the deletion of the DNA flanking the breakpoint, including one copy of the repeat sequences used by this mechanism.



### 4.3 DSB Repair Hierarchy

In normal cells, a functional hierarchy, regulated by an extensive cellular signalling network including cell cycle and DNA damage response factors, determines the use of the DSB repair pathways described above in order to ensure the efficient and faithful processing of DSBs (Mansour et al. 2008). NHEJ usually predominates and suppresses Alt-EJ, HR and SSA pathways. In situations of NHEJ deficiency, most DSBs are repaired by Alt-EJ, although HR and SSA also contribute more strongly to DSB repair (Mansour et al. 2008). Interestingly, DSB repair has been found to be frequently switched to Alt-EJ in tumour cell lines of various origin (Kotter et al. 2014) as well as in primary bladder (Bentley et al. 2004) and head-and-neck tumour cells (Shin et al. 2006). It is tempting to speculate that this switch to a more mutagenic repair mechanism enables tumours to accelerate their genetic diversification in order to overcome further barriers to growth. In any case, this switch to Alt-EJ in tumours offers opportunities for their specific targeting (Kotter et al. 2014).

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## 5 The DNA Damage Response as a Target for Tumour Radiosensitisation

Treatment outcomes could be improved for many tumours treated with radiotherapy if it were possible to selectively enhance tumour cell radiosensitivity without affecting normal tissue responses. Our progress in understanding the cellular radiation response over the past years sets the scene for the development of such strategies, based on targeting signalling and repair pathways that tumours have become addicted to.

### 5.1 The Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR) is a very important signalling factor in many tumour cells. It is frequently mutated or over-expressed in tumours and can be targeted therapeutically using antibodies such as cetuximab or tyrosine kinase inhibitors such as erlotinib or gefitinib, either as a monotherapy or combined with irradiation (Krause and Van Etten 2005). Combined treatment with cetuximab and radiotherapy has been demonstrated to improve local tumour control in patients with advanced head-and-neck cancer (Bonner et al. 2006). Interestingly, it was especially patients suffering strong cetuximab-associated side effects who benefitted most from the combined treatment. This suggests that there are subgroups of patients with a differential response to this targeted therapy (Bonner et al. 2010).

Cetuximab is thought to radiosensitise tumours at the cellular level (Harari et al. 2007). Although the exact mechanisms are not yet completely understood, DSB repair seems to be involved as it has been shown to be regulated by EGFR and to be suppressed by treatment with cetuximab or tyrosine kinase inhibitors which appear to block both NHEJ and HR (Kriegs et al. 2010; Myllynen et al. 2011). However,

the observed repair inhibition was not always associated with enhanced cellular radiosensitivity or improved tumour response (Myllynen et al. 2011; Kriegs et al. 2015; Stegeman et al. 2013). One possible explanation is that these treatments inhibit DSB repair only partially or transiently. Other responses observed following EGFR inhibition in combination with irradiation include the induction of apoptosis (though not confirmed in subsequent studies), semi-permanent cell cycle arrest and premature senescence which correlated with radiosensitisation (Kriegs et al. 2015; Wang et al. 2011). However, recent clinical trials report no improvement of therapy outcome for patients treated with tyrosine kinase inhibitors or cetuximab in combination with radiochemotherapy (Ang et al. 2014; Giralt et al. 2015; Martins et al. 2013; Mesia et al. 2015).

Apart from targeting EGFR function, its sheer abundance in tumours makes it also an ideal target for radioimmunotherapy, in order to selectively irradiate tumour cells (Cai et al. 2008; Saker et al. 2013), thereby allowing tumour control to be achieved with only moderate additional doses given with external beam radiotherapy (Koi et al. 2014). Other cell signalling factors that could be promising targets for DSB repair modulation include MAPK and AKT signalling (Kriegs et al. 2010; Toulany et al. 2006). Furthermore, EGFR-independent targets are being investigated, such as the proto-oncogenes Myc and Ras, and the therapeutic potential of multi-kinase inhibitors such as imatinib, dasatinib or sorafenib is being explored, with promising early results (Laban et al. 2013; Möckelmann et al. 2016). However, it is not at all clear at this stage whether DNA repair plays a significant role in these treatment strategies.

## 5.2 Poly (ADP-Ribose) Polymerase-1 (PARP1)

PARP1 contributes to a number of cellular tasks, such as DNA repair, replication, transcription and cell cycle regulation. In cancer therapy, it has been identified as an interesting druggable target because of its role in detecting DNA single-strand breaks and facilitating their repair via BER. Following PARP inhibition, single-strand breaks accumulate, resulting in the formation of DSBs especially during replication, which in turn require HR to be efficiently repaired. For this reason, HR-deficient tumours such as those carrying mutations in BRCA1 or BRCA2 have been targeted using PARP inhibitor. The logic behind this ‘synthetic lethality’ approach is that normal cells will tolerate PARP inhibition because they are HR-proficient, whereas tumour cells which are already compromised in HR, the backup repair pathway for unrepaired single-strand breaks encountered during replication, will struggle to survive upon loss of their main single-strand break repair pathway. PARP inhibition using drugs such as olaparib has been and is being used as a monotherapy in a number of studies which focus mostly on BRCA-deficient tumours (Bryant and Helleday 2004; Benafif and Hall 2015; Mateo et al. 2015). However, the combination of PARP inhibition with DNA-damaging chemo- or radiotherapy is even more promising, due to their synergistic interaction which has been shown *in vitro* as well as *in vivo* (Bryant et al. 2005; Fong et al. 2009; Tutt et al. 2010). Given that tumour sensitisation by PARP inhibition

is achieved mostly through the formation of DSBs at collapsed replication forks following their collision with unrepaired single-strand breaks, one would expect the best radiosensitising effects of PARP inhibition in tumours with a large S-phase fraction (Noel et al. 2006).

In addition to the ‘BRCAness’-dependent effects described above, it is becoming increasingly clear that also tumours that have switched from DNA-PK-dependent NHEJ to PARP-dependent Alt-EJ may also be promising candidates for tumour-specific radiosensitisation by PARP inhibition (Wang et al. 2003; Audebert et al. 2004; Mansour et al. 2010; Kotter et al. 2014).

### 5.3 Cell Cycle Checkpoint Signalling

Following irradiation, cells arrest at the G1/S and G2/M cell cycle checkpoints to allow time for DNA repair before cells enter the most critical phases of the cell cycle, namely replication and mitosis. In addition, an intra-S-phase checkpoint functions to postpone DNA replication in the presence of DNA damage (Morgan and Lawrence 2015).

DNA damage-induced cell cycle checkpoints are regulated by the kinases ATM and ATR via phosphorylation of the checkpoint kinases Chk2 and Chk1, respectively, which inhibit CDC25 phosphatases and thereby block cyclin-dependent kinase-mediated cell cycle progression. The initiating signals for these signal cascades are either a DSB which activates ATM or an extended stretch of single-stranded DNA coated with RPA (often associated with replication fork stalling and HR intermediates) which activates ATR (Marechal and Zou 2013). Accordingly, the G1/S and G2/M checkpoints are regulated by ATM/Chk2, whilst ATR/Chk1 are essential for the intra-S checkpoint and also contribute to the G2/M arrest. The G1/S checkpoint also requires an intact p53 response and downstream p21/CDKN1A induction and is therefore frequently compromised in tumours. In the absence of a functional G1/S arrest, p53-deficient tumours are very reliant on the ATR/Chk1 pathway to prevent entry into mitosis with too much DNA damage and may therefore be targeted using ATR or Chk1 inhibitors in combination with DNA-damaging agents such as radiotherapy, exploiting the synthetic lethality concept. Indeed, early studies have demonstrated enhanced sensitivity to radio- or chemotherapy of human cell lines derived from various tumour entities (Garrett and Collins 2011; Dillon et al. 2014; Busch et al. 2013).

However, toxicity and lack of specificity of the first generation of inhibitors, such as the Chk1 inhibitor UCN01, has been a serious drawback. More specific and better tolerated inhibitors for the checkpoint kinases, such as SCH 900776/MK-8776 or LY2606368, have been developed more recently and are now being tested in the clinic. Inhibitors for ATR and Wee1—which inhibits CDK1 and thereby delays entry into mitosis—are also being tested in phase I and II trials in combination with chemotherapy. Unfortunately, there are to date only two trials testing inhibitors for the G2/M arrest in combination with radiotherapy (NCT02223923, NCT01922076). As the targeted proteins are also involved in other

important aspects of DNA maintenance, such as repair and replication (Sorensen and Syljuasen 2012), their inhibition is also being explored as a monotherapy (McNeely et al. 2014). Furthermore, one could argue that any risk of systemic toxicity of these inhibitors when used in combination with chemotherapy may potentially be avoided or at least reduced when combining them with local radiotherapy treatments.

## 5.4 Cancer Stem Cells

The concept of cancer stem cells (CSCs) is based on that of normal tissue stem cells. Accordingly, CSC have the potential to self-renew, differentiate and maintain tumour growth and repopulation following radio- or chemotherapy. Two models exist for the organisation of CSC: one that assumes a hierarchically organised system in which only a small proportion of tumour cells actually have any tumourigenic potential whilst most tumour cells are unable to induce tumours (Lapidot et al. 1994; Al-Hajj et al. 2003), and a clonal evolution model in which random changes enable subclones to emerge with new functions and treatment responses within the tumour. In fact, both models may have some merit and could coexist, assuming that clonal evolution may shape the make-up of the small proportion of stem-like cells (Maugeri-Sacca et al. 2014).

Similar to tissue stem cells, CSC are generally thought to be resistant to DNA-damaging agents thanks to upregulated DNA damage response and repair pathways (Maynard et al. 2008; Mandal et al. 2011). Examples include CD133+ glioma stem cells which were shown to have an enhanced Chk1-dependent checkpoint response (Baumann et al. 2008; Bao et al. 2006) and upregulated expression of Nibrin, one component of the MRN complex which is involved in DSB sensing (Cheng et al. 2011). Enhanced DNA damage responses and repair gene expression were also observed in CD133+ CSC in A549 human lung carcinoma cells (Desai et al. 2014), mammary tumour-initiating cells (Zhang et al. 2014), pancreatic putative CSC (Mathews et al. 2011) and patient-derived non-small-cell lung cancer CSC (Bartucci et al. 2012). However, a number of studies showed no difference or even deficient DNA damage responses in CSC (McCord et al. 2009; Ropolo et al. 2009; Lundholm et al. 2013). These conflicting observations suggest that an enhanced DNA damage response may not be a general feature of all CSC and that inter-tumour and possibly even intra-tumour heterogeneity in the DNA damage response functionality may need to be considered (Magee et al. 2012). One important factor that may affect the composition and treatment response of CSC is epithelial–mesenchymal transition (EMT) which has been associated with enhanced radioresistance of mammary CSC. ZEB1, a zinc finger transcription factor which is phosphorylated and stabilised by ATM following DNA damage and then stabilises Chk1, appears to be an important player linking EMT to the DNA damage response (Zhang et al. 2014).

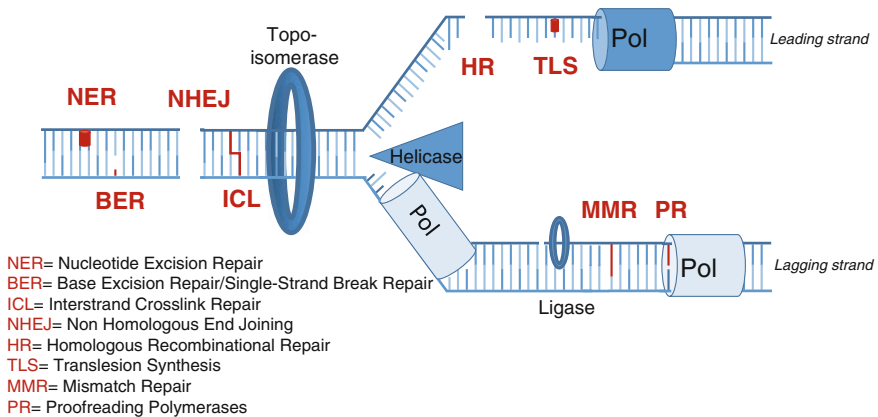
Interestingly, PARP1 inhibition was reported to radiosensitise glioma stem cells (Venere et al. 2014) and Chk1 inhibition reduced the CSC pool in non-small-cell

lung cancer cells, suggesting that DNA damage response inhibitors are a promising targeting strategy for CSC (Bartucci et al. 2012). However, as multiple DNA damage response pathways may be simultaneously upregulated in CSC, as shown in glioma stem cells, a multi-targeting approach involving combined inhibition of DNA repair and cell cycle checkpoint mechanisms may be a more promising strategy for overcoming CSC resistance (Signore et al. 2014; Ahmed et al. 2015).

## 5.5 Replication

One of the most exciting emerging strategies for improving tumour control is the replication-dependent sensitisation of tumours to radiation and chemotherapeutic drugs. It exploits the fact that DNA damage response pathways are of critical importance for replication fork stability, control of origin firing and the resolution of collapsed forks caused by DNA damage (Zeman and Cimprich 2014) (Fig. 2). They serve to counteract replication stress and the formation of secondary DSB induced by cytotoxic cancer therapies (Kotsantis et al. 2015).

The molecular targeting approaches for a range of DNA damage response pathways that are now becoming available for clinical testing provide the opportunity to enhance the toxicity of radio- and chemotherapy during replication (Pearl et al. 2015). Inhibition of the DNA damage response kinases ATR, CHK1 and WEE1 increases the activity of cyclin-dependent kinases, which leads to uncontrolled replication origin firing and depletion of the nucleotide pool, resulting in the



**Fig. 2** DNA repair mechanisms active at replication forks to maintain genomic stability. Cells are constantly exposed to insults from endogenous and exogenous agents that can introduce DNA damage and generate genomic instability. Many of these lesions cause structural damage to DNA and can alter or eliminate fundamental cellular processes, such as DNA replication. To counteract harmful effects, cells have developed a highly specialised DNA repair system, which can be subdivided into distinct mechanisms including base excision repair/single-strand break repair, nucleotide excision repair, mismatch repair, interstrand cross-link repair and double-strand break repair, together with the proofreading activity of polymerases and translesion synthesis

accumulation of replication-associated DSBs (Syljuasen et al. 2015). HR proteins which are not only involved in the repair of replication-associated DSBs but also support replication fidelity are also interesting targets (Huang and Mazin 2014). Examples include Rad51-, NUCKS- and RAD51-associated protein 1 whose disruption compromises replication fork progression, increases the firing of replication origins and negatively affects genome stability (Parplys et al. 2014, 2015a, b).

Inhibition of the DNA single-strand break repair factor PARP was first described as a promising strategy for BRCA-deficient mammary cancers, which, due to the associated HR deficiency, cannot efficiently deal with unrepaired DNA damage present during replication. However, PARP1 also senses stalled replication forks and recruits Mre11 which degrades the stalled forks to enable HR-dependent repair and replication fork restart (Ying et al. 2012).

ATR, CHK1 and PARP1 have been shown to be prime targets for radiosensitisation of S-phase cells (Ahmed et al. 2015; Dungey et al. 2008; Pires et al. 2012; Dobbstein and Sorensen 2015). Furthermore, irradiated cells undergoing replication form secondary DSBs (Groth et al. 2012) and suffer from blocked replication elongation (Parplys et al. 2012). For these reasons, radiation-induced tumour cell killing could potentially be enhanced by enriching the fraction of cells that are in S phase during irradiation, e.g. through the use of replication inhibitors (Dobbstein and Sorensen 2015).

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## 6 Biomarkers of Treatment Response

Radiotherapy treatment decisions are currently made based on clinical parameters such as tumour size, site and grade. The individual response of patient and tumour, based on their biological make-up, is typically not taken into account, simply because it has not been possible to reliably predict either the tumour or the normal tissue response of a particular patient, except for very rare cases of patients with radiation hypersensitivity syndromes such as ataxia telangiectasia. However, it would be of great benefit if it could be determined prior to the treatment which patients would actually benefit from radiotherapy, what dose should be given and whether the chances of tumour control could be improved using a particular molecular-targeted approach in combination with radiotherapy. Biological markers including mutational or single nucleotide polymorphism (SNP) signatures, gene expression profiles and functional assays for core radiation response mechanisms such as DSB repair capacity are slowly gaining relevance and are starting to pave the way for a precision medicine approach in radiation oncology.

We have reviewed the use of chromosome and DNA damage/repair assays for assessing individual radiation exposures and for predicting normal tissue responses quite extensively in the recent past (Chua and Rothkamm 2013; Pernet et al. 2012; Manning and Rothkamm 2013; Rothkamm et al. 2015) and will therefore focus on biomarkers of tumour response here.

Early attempts to use functional assays for tumour radiosensitivity prediction involved cell suspensions obtained from tumour biopsies which were irradiated *in vitro* and plated for colony formation to produce survival curves. Cell survival measured *in vitro* with such an approach was shown to correspond with clinical outcome in cervical cancer (West et al. 1993) and other tumour entities (Bjork-Eriksson et al. 2000), thus demonstrating that therapy outcome can be linked to cellular radiosensitivity. However, due to the amount of effort required for this assay and the long delay before colony counts are available, this method proved unsuitable for routine use in a clinical setting.

A quicker method for assessing the radiation response of tumour cells exploits the fact that cellular radiosensitivity is closely linked to DSB repair capacity. Immunofluorescence microscopic scoring of gamma-H2AX and/or 53BP1 nuclear protein foci, which form at the site of a DSB, is widely used as a surrogate marker of DSBs (Rothkamm et al. 2015) and can be performed in cell lines as well as in frozen or formalin-fixed paraffin-embedded tissue sections (Somaiah et al. 2012; Chua and Rothkamm 2013; Qvarnstrom et al. 2004; Barber et al. 2006; Crosbie et al. 2010; Rothkamm et al. 2012). This method has recently also been applied to determine residual DSBs in xenografts or patient-derived tumour biopsies following *in vivo* as well as *ex vivo* irradiation and repair incubation (Menegakis et al. 2015). A recent study demonstrated that residual foci levels obtained with this assay are consistent with the known differences in radioresponsiveness of different tumour types, thus providing proof of concept for this strategy (Menegakis et al. 2015). Additional information about the functionality of DNA damage response pathways in a tumour can be obtained using other biomarkers. For example, Rad51 foci formation following *ex vivo* treatment can indicate the functionality of the HR pathway and has been used to identify individual breast tumours with HR deficiencies (Naipal et al. 2014). As already mentioned above, patients with such tumours could then benefit from targeted therapy using PARP inhibitor. Biomarkers for other pathways, e.g. for a switch from classical to alternative end-joining, are currently being investigated.

In summary, functional *ex vivo* assays of biological radiation effects and pathway functions in tumour biopsies are rapid indicators of treatment response which enable treatments to be tailored to the individual characteristics of a tumour. Assaying function, rather than just genetic or expression profiles of a tumour, has the clear advantage of an integrated approach that will register effects, such as epigenetic or post-translational alterations, that may well be missed when using a non-functional method. However, for the assays to be fit for routine use in the clinic, robust, standardised procedures for sample logistics, processing and analysis need to be established and regularly validated.

Aberrant expression of a protein can also indicate whether a particular tumour will respond to a specific treatment strategy. Examples include HPV-positive oropharyngeal cancers which over-express p16/INK4a (Lassen et al. 2009), head-and-neck cancers deficient in the DNA damage response due to downregulated ATM (Mansour et al. 2013), over-expression of EGFR associated with head-and-neck cancer radioresistance (Ang et al. 2002), prostate cancer cells which



over-express Bcl2, causing them to use alternative instead of classical end-joining (Catz and Johnson 2003; Wang et al. 2008), or colorectal carcinoma with deregulated HR and over-expression of RAD51 (Tennstedt et al. 2013). Also, over-expression of Ku, but possibly not of other players involved in NHEJ, was found to be associated with radioresistance in head-and-neck cancer (Lee et al. 2005; Moeller et al. 2011).

As gene expression profiling and whole-exome sequencing are becoming more affordable as well as accessible, there is increasing interest in identifying signatures that could be used to predict treatment response and individualise treatment. However, to date, no reliable signatures are available for routine use, although some candidate profiles have been reported (Ahmed et al. 2015; Tinhofer et al. 2015; Spitzner et al. 2010; Pramana et al. 2007).

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## 7 Future Perspectives

Major progress has been made in recent years in the investigation and characterisation of cellular and molecular DNA damage response processes occurring in irradiated tumours and normal tissues. One important driver has been the development of new cellular and molecular methods and techniques. These have facilitated exciting discoveries at the cellular level, especially in the fields of signal transduction, cell cycle regulation and DNA repair. The recent discoveries should not only further our understanding of the cellular response to ionising radiation, but they may also help us develop and refine cancer treatment strategies. For instance, we now understand much better the opportunities—but also the potential caveats—that need to be considered when targeting signalling cascades, such as those involving EGFR, MAP kinase or the mTOR/AKT pathway, to selectively inactivate and/or sensitise tumour cells.

New discoveries have especially been made in the field of DNA repair. They now provide the exciting opportunity to understand the biochemical basis which underpins the large variation of tumour cell radiosensitivity that we have grappled with for so long. Tumour cells frequently suffer from deregulated DNA double-strand break repair, such as a switch from classical to PARP-dependent alternative end-joining or a ‘BRCAness’ phenotype of HR deficiency, which provides new targets for the selective sensitisation of tumours. The first successful steps in this direction involve the blocking of PARP or the checkpoint factors Chk1 and Chk2.

Gene, miRNA or protein expression profiling as well as functional assays should provide the means to assess the individual radiosensitivity, DNA repair capacity and susceptibility of a tumour to specific targeted therapies. However, overall, one should not underestimate the complexity of the interrelationship between DNA repair, other cellular processes and microenvironmental factors which will necessitate a careful evaluation of any initial findings.



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# Cancer Stem Cells

Wendy A. Woodward and Richard P. Hill

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## Abstract

The cancer stem cell model in solid tumors has evolved significantly from the early paradigm shifting work highlighting parallels between the stem cell hierarchy in hematologic malignancies and solid tumors. Putative stem cells can dedifferentiated, be induced by context, and be the result of accumulated genetic mutations. The simple hypothesis that stem cell therapies will overcome the minority of cells that lead to recurrence has evolved with it. Nevertheless, the body of evidence that this field is clinically relevant in patients and patient care has grown with the complexity of the hypotheses, and numerous clinical strategies to target these cells have been identified. Herein we review this progress and highlight the work still outstanding.

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## Keywords

Cancer stem cells · Stem cell markers · Stem cell models · Stem cell resistance · Microenvironment

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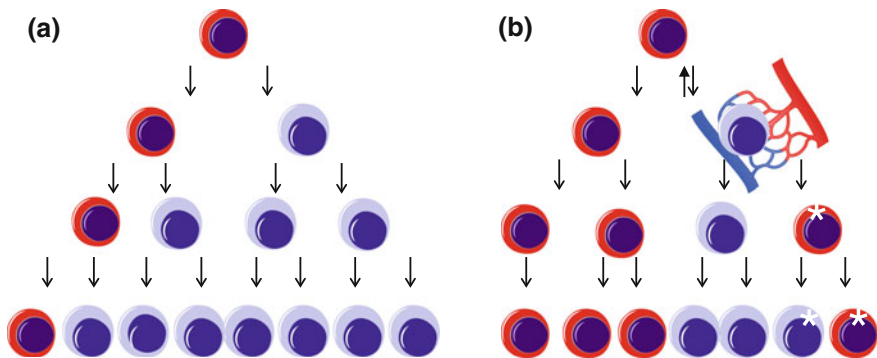
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## 1 Overview of the Evolution of the Cancer Stem Cell Model

Work over the last decade has highlighted the potential importance of stem cell populations in tumors—cancer stem cells (Clarke et al. 2006; O’Brien et al. 2009). Such cells (CSCs) have been argued to represent the critical population for predicting progression and treatment outcome presuming that their number and treatment sensitivity are important for tumor control by radiation and chemotherapy. The prospective demonstration that only small specific populations of cells, derived from a bulk solid tumor population based on expression of specific surface markers, recreated human tumors in outgrowth experiments propelled the subsequent 10 years of cancer stem cell research. The initial concept was that tumors are organized as steep hierarchies from which only a small percentage of cells are capable of self-renewing and recapitulating the tumor heterogeneity. This concept built on normal stem cell data attributing characteristics of normal stem cells—multipotency, unlimited replication potential, and self-renewal—to proposed cancer stem cells. The cancer stem cell hierarchy was initially viewed as a largely rigid top-down progression from the most primitive cancer stem cells at the top to the most differentiated bulk cells at the bottom. It was presumed that individual cancer stem cells reproduced themselves to maintain self-renewal and as needed produced differentiated daughter cells to maintain homeostasis with a small population of cancer stem cells (Fig. 1a). This model presumed the functional and phenotypic differences of stem cells versus differentiated cells were independent of genetic mutation, mediated instead by epigenetics and differentiation commitment. This stood somewhat at odds with the clonal dynamic, driver mutation model of tumor



**Fig. 1** Increasing complexity in the cancer stem cell model. Drawing from parallels in hematopoietic development, prospective isolation of tumor-initiating cells from solid tumors led to early models of cancer hierarchy similar to the normal state (1A). Primitive, self-renewing cells were presumed to maintain the tumor bulk and the minority population of cancer stem cells. In the last decade has been demonstrated that context and microenvironment can promote tumor initiation, that mutations (\*) can confer self-renewing capacity, that some tumors become predominantly composed of self-renewing cells, that markers of self-renewing cells are context dependent, and that functional initiating cells can be both genetically similar or dissimilar (Fig. 1b)

progression and recurrence; the evidence for both was hotly debated. Early on, there were no studies merging genetic analyses with *stemness* studies, and very little consideration was given to the role that context might have in influencing the stem cell population. Over time, however, many new data emerged, challenged the initial paradigm, and were incorporated into this initially simplistic model (Fig. 1b).

After the reports of the first solid tumors to apparently be organized in a cancer stem cell hierarchy (Al-Hajj et al. 2003; Singh et al. 2004), it was demonstrated that the cancer stem cell compartment size and depth of hierarchy depend on tumor type and that self-renewal assays of tumor regrowth in transplants predict for the biology associated with engraftment in animals, which might not faithfully capture the biology of recurrence in situ (Feuring-Buske et al. 2003; Quintana et al. 2008; Notta et al. 2010; Rehe et al. 2013). New markers and strategies to prospectively identify stem cells emerged (Collins et al. 2005; Bao et al. 2006; Dalerba et al. 2007; Ginestier et al. 2007; Hermann et al. 2007; Li et al. 2007; O'Brien et al. 2007; Patrawala et al. 2007; Prince et al. 2007; Ricci-Vitiani et al. 2007; Eramo et al. 2008; Curley et al. 2009; Li et al. 2009a; Piccirillo et al. 2009; Stewart et al. 2011; Wang et al. 2011; Chen et al. 2012; Charafe-Jauffret et al. 2013; Wu et al. 2013; Zhang et al. 2015), and from these, a series of prognostic signatures were derived across tumor types [Table 1 (Gentles et al. 2010; Eppert et al. 2011; Merlos-Suarez et al. 2011; Bartholdy et al. 2014)]. This connection to clinical outcome was reassuring that the cancer stem cell model was relevant. However, the clinical complexity and challenges to incorporation into clinical management were clearly illuminated by findings from patients that the prospectively identified populations that maintain outgrowth potential in tumors may be different in different tumors and patients (Eppert et al. 2011). This further highlighted the need to move to the use of functional demonstrations of stemness rather than the use of markers that are promiscuous, often not linked to function, are potentially transient and depend on context. To this end, the inducible lineage-tracing and re-tracing experiments were developed in genetically engineered mouse models to overcome these issues [reviewed in Roy et al. (2014)], and in some cases, these validated the stem cell model, but they still have some limitations as discussed in more detail below.

Clonal dynamic studies using lineage-tracing approaches in normal tissues demonstrated some common themes across tissues in some cases. In gut and skin, maintaining the frequency of stem cells during homeostasis appeared not as a function of asymmetric division of the primitive stem cell, to create one stem cell and one daughter cell, but rather through maintenance at the population level [reviewed in Blanpain and Simons (2013)]. In other studies, the stem cells identified in lineage-tracing experiments did not align with the prior findings from transplantation experiments, suggesting that transplantation assays may provide circumstances that permit or promote tumor initiation that would not occur in situ. For example, the first lineage-tracing experiments to define cell fate in the developing mammary gland demonstrated that the bipotent differentiation potential of single cells described after transplantation is not identified in situ (Van Keymeulen et al. 2011; van Amerongen 2014). Instead two unipotent basal and luminal stem cells were identified. The same was described in prostate development (Liu et al. 2011;

**Table 1** Tumor-initiating cell-related gene signature studies reporting prognostic signatures in independent patient data derived from bulk cells (Glinsky et al. 2005; Phillips et al. 2006; Liu et al. 2007; Shipitsin et al. 2007; Stevenson et al. 2009; Gentles et al. 2010; Eppert et al. 2011; Merlos-Suarez et al. 2011; Becker et al. 2012; Liu et al. 2012; Atkinson et al. 2013; Metzeler et al. 2013; Schwede et al. 2013; Van den Broeck et al. 2013; Peng et al. 2014; Yin et al. 2014; Pfefferle et al. 2015; Yang et al. 2015)

Cancer Type	Signature Source
	<b>Genes identifies in minority stem-like population prognostic in independent tumor samples</b>
Breast, brain, lung prostate	Liu et al. (2007). The prognostic role of a gene signature from tumorigenic breast cancer cells.
Breast	Shipitsin et al. (2007). Molecular definition of breast tumor heterogeneity.
Breast (H2N+)	Liu et al. (2012). Seventeen-gene signature from enriched Her2/Neu mammary tumor-initiating cells predicts clinical outcome for human HER2+: ERalpha- breast cancer.
Breast	Yin et al. (2014). A 41-gene signature derived from breast cancer stem cells as a predictor of survival.
Colon	Merlos-Suarez et al. (2011). The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse.
Pancreas	Van den Broeck et al. (2013). Human pancreatic cancer contains a side population expressing cancer stem cell-associated and prognostic genes.
Leukemia	Gentles et al. (2010). Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia.
Leukemia	Eppert et al. (2011). Stem cell gene expression programs influence clinical outcome in human leukemia.
Leukemia	Metzeler et al. (2013). A stem cell-like gene expression signature associates with inferior outcomes and a distinct microRNA expression profile in adults with primary cytogenetically normal AML.
Leukemia	Yang et al. (2015). Systematic computation with functional gene-sets among leukemic and hematopoietic stem cells reveals a favorable prognostic signature for acute myeloid leukemia.
	<b>Genes extracted based on embryonic or developmental correlation prognostic in independent tumor samples</b>
Breast	Pfefferle et al. (2015). Luminal progenitor and fetal mammary stem cell expression features predict breast tumor response to neoadjuvant chemotherapy.

(continued)

**Table 1** (continued)

Cancer Type	Signature Source
Prostate, breast, lung, ovarian, bladder, lymphoma, mesothelioma, brain, and leukemia	Glinsky et al. (2005). Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer.
Lung	Stevenson et al. (2009). Characterizing the clinical relevance of an embryonic stem cell phenotype in lung adenocarcinoma.
Liver	Becker et al. (2012). Genetic signatures shared in embryonic liver development and liver cancer define prognostically relevant subgroups in HCC.
Brain	Phillips et al. (2006). Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis.
Ovary	Schwede et al. (2013). Stem cell-like gene expression in ovarian cancer predicts type II subtype and prognosis.
Prostate	Peng et al. (2014). An expression signature at diagnosis to estimate prostate cancer patients' overall survival.
	<b>Genes identified in minority stem-like population prognostic in independent normal breast samples from patients with tumor</b>
Breast	Atkinson et al. (2013). Cancer stem cell markers are enriched in normal tissue adjacent to triple negative breast cancer and inversely correlated with DNA repair deficiency.

Wang et al. 2014). Later, Rios et al. highlighting the potential caveats of these approaches identified a single bipotent stem cell in the mammary gland (Rios et al. 2014). Speculation regarding contributors to the dramatic differences in the results of these studies includes differences in promoter specificity and/or transcriptional activity due to approaches used, labeling efficiency in different lineages, and confocal imaging variation across the studies [reviewed in Oakes et al. (2014)]. Alternatively or in addition, it may simply reflect the heterogeneous results of a legitimately complex system revealed through differing studies.

Fate mapping was carried out in tumors including benign papilloma and squamous cell carcinoma, glioma, and intestinal adenomas (Chen et al. 2010; Driessens et al. 2012; Schepers et al. 2012). Progression in skin cancer appeared to track with a decrease in the steepness of the hierarchy (Driessens et al. 2012). Through the fate mapping/lineage-tracing experiments as well as with the use of animal models, it was additionally demonstrated that the stem cell frequency could be altered by genetic mutations in the stem cells or background (Vaillant et al. 2008; Curtis et al. 2010; Vermeulen et al. 2013) and that for some normal tissues and many tumors, the pool of stem cells could be replenished, if significantly depleted, via dedifferentiation of a previously non-self-renewing cell (Debeb et al. 2012; van Es et al. 2012;

Buczacki et al. 2013; Schwitalla et al. 2013). The latter challenged the idea that targeting cancer stem cells within bulk tumors would be curative since the remaining more differentiated cells could potentially replace the targeted pool and might be driven to do so by a shift in stem-differentiated cell equilibrium caused by therapy targeting one side of the equation. This demonstration of plasticity led to the concept that stem cells in fact represent a heterogeneous compartment into which cells readily transit and exit becoming temporarily primed for specific stem cell activity [reviewed in Blanpain and Simons (2013)]. It was clear that pressure on the tumor cells such as therapy could shift this equilibrium and that specific signaling pathways could be identified that mediated these transitions.

The most plastic of tumor cells were also presumed to transition between epithelial and mesenchymal states to escape from the primary soil into the circulation and beyond to reseed distant soil (Liu et al. 2014). It further became clear that the microenvironment, including a niche of cells that supported the stem cell state, contributed to maintaining this proposed transient stemness compartment [reviewed in Inman et al. (2015)]. As numerous normal cells were identified as niche conspirators, including macrophages and mesenchymal stem cells, distinct niches for active versus quiescent or dormant stem cells were proposed (Ehninger and Trumpp 2011), and subsequently, the possibility of end organ-specific niches, bone marrow versus lung versus brain, was added to the emerging complex picture.

Alongside the progress made through lineage-tracing experiments, progress in genetic analysis began to converge on the cancer stem cell field. Studies merging these fields led to a direct demonstration that the pressure of therapy to select surviving clones indeed did not in all cases select genetically hardy clones, but rather phenotypically hardy clones, supporting the cancer stem cell hypothesis (Kreso et al. 2013). It was further shown in this work transplanting 150 single cells from 10 colorectal cancer patients that there can be genetic variability within a clone derived from a single stem cell (Kreso et al. 2013). Kreso and Dick proposed a unified model drawing on the genetic and cancer stem cell data and hypothesized that the accumulation of cancer-promoting mutations in the most primitive normal stem cells at the top of the hierarchy led to the most undifferentiated and aggressive cancers, while mutations in more differentiated cells might confer self-renewal and therefore lead to less aggressive cancers (Kreso and Dick 2014). Consistent with this proposal, Tomasetti and Vogelstein report a strong correlation extending over five orders of magnitude between lifetime incidence of multiple cancers and the estimated number of normal stem cell divisions in the corresponding tissues over a lifetime. This suggests that random errors occurring during DNA replication in normal stem cells are a major contributing factor in cancer development (Tomasetti and Vogelstein 2015). Without question, the cancer stem cell model has matured making room for greater complexity.

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## 2 Markers and Models

As above, the solid tumor cancer stem cell field was propelled forward by landmark papers in which tumorigenic breast and brain cancer cells were prospectively identified and distinguished from non-tumorigenic cells in the same cancer using

membrane markers (Al-Hajj et al. 2003; Singh et al. 2004). The readout in these studies was tumor outgrowth in an orthotopic xenograft. This work led to a rapid increase in papers across many tumor types identifying marker sets that prospectively identified the tumorigenic population in human tumors and cell lines using outgrowth in a xenograft as the proof of stemness (Collins et al. 2005; Bao et al. 2006; Dalerba et al. 2007; Ginestier et al. 2007; Hermann et al. 2007; Li et al. 2007; O'Brien et al. 2007; Patrawala et al. 2007; Prince et al. 2007; Ricci-Vitiani et al. 2007; Eramoi et al. 2008; Curley et al. 2009; Li et al. 2009a; Piccirillo et al. 2009; Stewart et al. 2011; Wang et al. 2011; Chen et al. 2012; Charafe-Jauffret et al. 2013; Wu et al. 2013; Zhang et al. 2015) (Table 2). In sum, these studies demonstrated minority tumorigenic populations in multiple tumor types including breast, colon, pancreas, head and neck, sarcoma, lung, ovary, AML, and CML. These studies relied on immunocompromised mice to grow human tumors, and it was quickly recognized that mice with greater immune suppression yielded higher frequencies of tumorigenic cells in AML, ALL, melanoma, and lung cancer (Quintana et al. 2008; Taussig et al. 2008; Chiu et al. 2010; Ishizawa et al. 2010; Notta et al. 2011) raising the question of whether the apparent tumor hierarchy was an artifact of the assay or a clinical reality, although studies supporting the reality were also compelling (O'Brien et al. 2009; Ishizawa et al. 2010). It was further noted that not all murine growth factors cross-react with human receptors and that numerous tissue processing issues may impact the outgrowth in a transplantation assay (Bossen et al. 2006; Rongvaux et al. 2013). One approach to address the variability related to altered immunity was to examine tumors in syngeneic mice with intact immune systems. Consistent with the data from the human tumors, several tumor types examined in these studies supported the cancer stem cell model (Neering et al. 2007; Vaillant et al. 2008; Zhang et al. 2008; Read et al. 2009; Ward et al. 2009). These studies did not necessarily yield markers that are relevant in human cancers however, a difference may relate to the fact that many surface markers do not relate directly to stem cell function.

Following the identification of markers in various solid tumor types, there were numerous studies using these markers in vitro and in translational work to identify genetic signatures from these populations, to identify targets to eradicate them, and

**Table 2** Markers reported to prospectively identify tumor initiation from human tumors

<b>Prostate</b>	<b>CD44+</b>		
<b>Head and Neck</b>	<b>CD44+</b>	<b>SP</b>	
<b>Breast</b>	<b>CD44+ ESA+ CD24-</b>	<b>ALDH</b>	<b>GD2</b>
<b>Colon</b>	<b>CD44+ ESA+</b>	<b>CD133+</b>	<b>CD166</b>
<b>Pancreas</b>	<b>CD44+ ESA+ CD24+</b>	<b>CD133+ CXCR4+</b>	
<b>Glioma</b>	<b>CD133+*</b>		
<b>Lung</b>	<b>CD133+</b>		
<b>Ovary</b>	<b>CD133+</b>	<b>CA125</b>	

<sup>a</sup>Controversial. Abbreviations: SP, side population; ALDH, aldehyde dehydrogenase activity; ESA, epithelial-specific antigen

*Blue* and *Green* colors denote their relevance across tumor types. *Bold* represent single markers

to demonstrate their relationship to prognosis. While these were supportive of the model in many cases, it was quickly demonstrated that the markers can depend on context [reviewed in Meacham and Morrison (2013)], that they are promiscuous, and that they are not necessarily related to function. In larger studies of patient samples, it was apparent that in some tumors, the tumorigenic potential may reside in varying minority populations, suggesting that functionally determining which cells were CSCs would need to be a component of individual patient sample analysis (Chiu et al. 2010; Eppert et al. 2011; Sarry et al. 2011). Certainly, it is clear stem cell markers identified and validated in one xenograft model cannot be assumed to identify CSCs in new systems or models where this has not been explicitly demonstrated. Further, it remains to be seen how widely results from cancer stem cell models will apply to the clinic, although various clinical studies have reported that the proportion of cells expressing CSC markers, such as ALDH1 or low proteasome activity, correlates with treatment outcome (Lagadec et al. 2014; Atkinson et al. 2013; Ginestier et al. 2007).

While marker studies furthered the field by identifying cells with tumorigenic potential under permissive circumstances, lineage-tracing studies including proliferation kinetics and clonal dynamics [reviewed in Blanpain and Simons (2013)] have allowed more direct examination of the clonal dynamics of the stem cells under more relevant contextual circumstances. Three techniques have been used to study proliferation kinetics in population-based assays. These are pulse-chase, continuous labeling, and label dilution experiments. These can be applied *in vivo* by targeting inducible reporter constructs with lineage-restricted promoters to a small number of cells and examining the distribution of labeled cells after elapsed time for the organ of interest to develop. Quantitative analysis is performed to assess the clonal dynamics based on the fixed tissue analysis. These approaches cannot definitively distinguish between population balance that is perfectly maintained through either asymmetric division of a single stem cell that results in a stem cell and a differentiated cell versus division of a stem cell into two stem cells. Importantly, although they have been used to demonstrate differences in multipotency among stem cells in their native context, further work to resolve the fate of individual cells is needed to determine whether lineage is specified early (bestowed on only a few cells early on) or instead involves a competition between equipotent precursors.

In the gut, lineage tracing identified two stem cell pools, one LGR5-expressing pool and a second BMI-1-expressing pool. It was further shown that on ablation of the LGR5 pool, the BMI-1 expressing stem cells can repopulate the crypt (Barker et al. 2007; Sangiorgi and Capecchi 2008; Barker et al. 2012). What is not clear, however, is whether these populations are mutually exclusive. Indeed recent studies have raised the possibility that this work may have targeted the same pool using different promoters (Itzkovitz et al. 2012; Munoz et al. 2012; Buczaccki et al. 2013), and Blanpain et al. speculate that the stem cell pool may express all of the identified markers at different times specified by different contexts (Blanpain and Simons 2013). Quantitative studies using these models demonstrated that the number of label-retaining cells was maintained over time by increase in the size of remaining clones as the total number of surviving clones diminished and largely ruled out the



likelihood that ingrained hierarchy accounts for self-renewal, demonstrating instead that neutral competition for limited access to the niche dominates this process (Lopez-Garcia et al. 2010; Snippert et al. 2010b). Similar to the findings in the gut, lineage tracing in the skin also revealed that clones are lost over time and that the constant label-retaining pool is accounted for at the population level by proliferation of the remaining pool (Clayton et al. 2007; Doupe et al. 2010). Quantitative studies here suggested that the tissue was maintained by a single progenitor population, which divided asymmetrically most of the time, but may also divide symmetrically or terminally differentiate to maintain balance. Studies of response after injury mentioned below in aggregate support the model in which the pool is maintained by progenitors and a slower cycling stem cell pool sit ready in response to injury (Ito et al. 2005; Levy et al. 2007; Jaks et al. 2008; Snippert et al. 2010a). The possibility that these progenitors revert into the slow-cycling stem cell pool as described in esophagus (Doupe et al. 2012) cannot easily be ascertained or ruled out. It was reported that location within the niche predetermines the likelihood of a given cell to remain uncommitted or to differentiate, but that committed cells can replenish the stem cell pool after depletion (Rompolas et al. 2013). Using genetic lineage-tracing strategies, similar dedifferentiation behavior as that described in the skin has been reported for the Delta-like 1-expressing cells in the mouse intestine where lineage tracing demonstrates these normally committed, differentiated cells can be recruited into the stem cell compartment if needed upon injury (van Es et al. 2012). Similarly committed Paneth cells can apparently repopulate the stem cell compartment when needed (Buczacki et al. 2013). This important role of position and context has also been demonstrated to regulate the proliferation or quiescence of cancer stem cells (Bissell and Inman 2008).

Fate mapping in tumors was similarly informative. Expression of a conditional reporter in a small population of benign papilloma cells confirmed a hierarchical organization, which became more shallow on progression to squamous cell carcinoma (Driessens et al. 2012). In intestinal adenomas, the previously identified stem cell marker *Lgr5+* was tracked through the development of benign lesions using a multicolor lineage reporter. The marked normal stem cells gave rise to the adenomas, and these cells in the adenoma contributed extensively to the tumor growth. The preponderance of *Lgr-* progeny led to the speculation that the *Lgr+* cells gave rise to largely non-proliferative *Lgr-* cells. Reflecting what is likely a clinical reality, similar studies of intestinal adenomas in different context yield a dissimilar story. Upon Wnt pathway activation, Vermeulen et al. found the *Lgr-* cells could contribute to the adenoma formation and *Lgr-* cells gave rise to *Lgr+* cells (Vermeulen et al. 2013). It has not yet been established what fraction of adenomas have a hierarchical organization, and how it relates to progression to invasive cancer has not been studied. In glioma studies, the presumptive *Nestin+* stem cell population was selectively depleted extending the animals' lives (Chen et al. 2012). Regrowth after therapy with temozolomide was attributed to the *Nestin+* population correlating this population to cancer stem cell status although it was not conclusively demonstrated that *Nestin-* cells did not contribute (Chen et al. 2012).

### 3 Role of the Tumor Microenvironment

The stromal components and cell–cell interactions in a tumor play an important role in its growth and response to treatment. Stroma within a tumor includes the vasculature, various populations of cells derived from the bone marrow (BMDC) such as monocytes/macrophages and a variety of immune cell populations, cancer-associated fibroblasts, and non-cellular tissue components such as collagens, fibronectin, and laminin. Further, the poorly organized structure of the vasculature in most tumors (Vaupel et al. 1989) creates an environment in which there is substantial heterogeneity in the supply of nutrients such as oxygen or glucose and in the removal of catabolic products. This leads to regions of low oxygen tension (hypoxia), high levels of acidity due to lactic acid production, increased interstitial fluid pressure due to increased leakiness of the blood vessels, and poor removal of tissue fluid partly caused by lack of functional lymphatics. Specific microenvironmental factors, but also cell–cell interactions and genetically regulated cellular signals, are important determinants for stem cell maintenance and survival. As discussed above, different kinds of ‘niches’ have been described in which certain stromal cell populations may provide a supportive environment for CSCs and/or help to maintain the stem-like phenotype of tumor cells (Pajonk and Vlashi 2013). For example, in two mouse models of metastatic breast cancer, distinct endothelial sub-niches were shown to regulate disseminated tumor cell dormancy with vascular homeostasis maintaining quiescence but stimulation of vasculature causing out-growth of the tumor cells (Bissell). It has also been reported that glioblastoma cells may sit in a perivascular niche involving endothelial cell contact (Heddleston et al. 2010) but it has also been reported that both glioblastoma and breast cancer CSCs may sit at a distance from functional vasculature and can be at low oxygen levels (i.e., in an hypoxic niche) (Heddleston et al. 2010; Liu et al. 2014; Peitzsch et al. 2014). Interestingly, hypoxia can suppress miRNA levels by repression of both the DICER and DROSHA enzymes, which are required for miRNA processing (van den Beucken et al. 2014). This leads to a significant decrease in overall levels of certain miRNA in hypoxic cells, which in turn can lead to the acquisition of stem and metastatic phenotypes. In a genetically engineered mouse model of soft tissue sarcoma, deletion of one allele of DICER can decrease miRNA expression and increase the rate of metastasis to the lung (Mito et al. 2013). In breast cancer, reduction in DICER results in a selective loss of the miR200 family of proteins, which stimulates an epithelial to mesenchymal transition (EMT) (van den Beucken et al. 2014). This transition has been associated with a CSC phenotype in breast cancer cells (Mani et al. 2008; Liu et al. 2014). Exposure to hypoxia has also been reported to result in changes in the methylation levels of certain genes, due to a requirement for oxygen by some of the enzymes that cause demethylation. This results in a more primitive phenotype similar to that of stem cells. Thus, exposure to hypoxia may cause various epigenetic changes that promote a stem cell phenotype.

## 4 Response to Therapy

There are many datasets which support a higher treatment resistance of CSC to both radiation and chemotherapeutic drugs compared to non-CSC (Krause et al. 2011; Alison et al. 2012; Sebens and Schafer 2012; Alisi et al. 2013; Holohan et al. 2013; Crowder et al. 2014; Rycaj and Tang 2014; Cui et al. 2015). The increased resistance to chemotherapy has been variably associated with the proliferative quiescence of CSCs and their resistance to DNA damage and reduced susceptibility to induction of apoptosis. A high expression of ABC transporters that can pump drugs out of cells has also been observed in CSCs. Early studies demonstrated an increase in the ex vivo fraction of CD133 positive cells, confirmed as CSC by transplantation assays, following in vivo irradiation of glioma xenografts. Interestingly, DNA damage checkpoints were preferentially activated in marker-positive versus marker-negative cells (Bao et al. 2006). Higher levels of antioxidant molecules have also been observed in CSCs suggesting increased ability to inactivate reactive oxygen species (ROS), a mediator of radiation damage in cells (Diehn et al. 2009). Compared to progenitor cells, breast CSCs have been shown in vitro to contain a lower level of ROS with higher expression of genes involved in ROS scavenging. Moreover, the initially higher post-irradiation clonogenic cell survival of breast CSC can be altered by pharmacological modulation of the ROS levels. Recent studies by Pajonk and colleagues have also reported that low proteasome activity is associated with a stem cell phenotype and that cells from tumors with low proteasome activity are more resistant to chemotherapy and radiation treatment (Lagadec et al. 2012, 2014; Vlashi and Pajonk 2015). However, a higher intrinsic resistance of CSC cannot be regarded as a general phenomenon, since heterogeneity seems to exist between individual tumors of the same histology (Zielske et al. 2011). Further, the early work of West et al. (West et al. 1993) reported a wide range of radiosensitivities for cells derived from different tumors of the cervix and head and neck that were capable of growing in in vitro clonogenic assays in agarose. This was a spheroid-like environment, although not the same as currently accepted stem cell assays.

A link between hypoxia and putative stem cells has also been shown by an increase in the fraction of CD133-positive cells in brain tumor cells exposed to hypoxia in vitro (Blazek et al. 2007; Platet et al. 2007) and the preferential expression of HIF2 $\alpha$ - and HIF-regulated genes in glioma stem cells (Li et al. 2009b). This might be expected to affect their relative radiosensitivity, although it should be noted that the level of hypoxia in 'hypoxic' niches is not well defined and may represent a level of hypoxia more consistent with increased levels of HIF-1 $\alpha$  and HIF-2 $\alpha$  (<~10–20 mm Hg) rather than the levels required for full hypoxia-induced radioresistance (<1–5 mm Hg) (Wilson and Hay 2011). Important in this context is that EMT (which as noted above can be induced by hypoxic exposure) has also been previously associated with increased radiation resistance (Theys et al. 2011; Bhat et al. 2013; Al-Assar et al. 2014; Zhang et al. 2014), as well as increased metastatic potential. The hypoxic niche has also been reported to

protect colon cancer CSCs from chemotherapy (Mao et al. 2013). However, a recent study has reported that hypoxia does not affect the radiation survival of breast cancer stem cells cultured as mammospheres putatively because of their high levels of antioxidant molecules capable of scavenging reactive oxygen species (Lagadec et al. 2012). Thus, the potential role of hypoxia in modifying the treatment sensitivity of CSCs *in vivo* remains uncertain and may vary from tumor to tumor.

The number of CSCs in tumor is highly heterogeneous but will also play an important role in overall response to treatment. In animal models, it was demonstrated that the number of (putative) stem cells assessed by transplantation assays correlated with the single radiation dose required for tumor control (Hill and Milas 1989; Baumann et al. 2008). Similar results have been reported for experimental studies in animal models using fractionated radiation treatment (Yaromina et al. 2007) and the expression of the stem cell-related marker CD44 has been reported to correlate with local control in early laryngeal cancers treated with radiation (Baumann and Krause 2010). Two important considerations in the context of the analysis of the treatment sensitivity of CSC are the increasing evidence for the plasticity of the cancer stem cell phenotype and the method of assessment. The concept that early progenitor cells may regain stem cell properties induced by treatment would result in an effective increase in the number of CSCs in the tumor and hence the level of cell killing required to achieve tumor control (Pajonk and Vlashi 2013; Vlashi and Pajonk 2015). The results of Bao et al. (2006) mentioned above, in which radiation-treated gliomas demonstrated increased CSC content, may be explained by the concept that the radiation treatment induced progenitor cell populations in the tumor to reacquire stem cell properties, consistent with findings in normal tissues described above concerning rebalancing of stem and progenitor cell populations after traumatic injury. Assessing the sensitivity of CSCs *in vivo* using an experimental tumor response assay to determine treatment outcome rather than tumor control assay is also a concern. The essence of the CSC model is that it is the killing of these cells that is ultimately responsible for tumor control, whereas tumor response can reflect the sensitivity of both stem cells and progenitor cells (Baumann et al. 2008). This concern makes it particularly difficult to use experimental studies to assess the response of CSC to drug treatment *in vivo*, since such treatments are rarely capable of achieving tumor control on their own, and thus, they reflect the response of both stem and progenitor cells. Combination treatments with radiation can potentially address this concern, and such studies have indicated the failure of certain drugs to target stem cell populations (Baumann/Krause). A further complication is that these two considerations are independent of one another but may, of course, both occur during tumor treatments *in vivo*; thus in some cases, the interpretation of studies assessing the treatment sensitivity of CSC may be impacted by factors that do not directly relate to the sensitivity of the individual tumor cells. *In vitro* studies of the drug or radiation sensitivity of cells expressing putative stem cell markers can partially overcome this concern but the different environments found within tumors require that such observations are confirmed by *in vivo* studies.

## 5 Conclusion

Without question, the cancer stem cell model has been refined numerous times in the last decade and is far more complex than initially proposed. Heterogeneity within tumors and even in clonal populations within tumors, between tumors in the same patient, and between patients as well as across tumor types are the common themes that emerge across fields. The prognostic value of signatures from small populations of cells may imply that there are ways to clinically identify patients whose tumors are driven by a stem cell phenotype that may be amenable to directed therapy and get around the challenges of variation in prospective markers between patients as well as the limited feasibility in profiling each tumor for stemness markers in order to make treatment decisions. Nevertheless, real-time tumor profiling will likely be needed to select patients for therapy, and to date, there are still no trials selecting patients for treatment using such approaches and strategies to merge stem cell targeting with genetics-based targets are in their infancy. Certainly, advances in clinical imaging-based identification of stem cell-driven tumors would greatly enhance the translatability of these models as would liquid biopsy advances still very much unexplored in this area. Still, in spite of the work yet to do, the progress in the last decade has been rapid and continues on.

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# Molecular Targeting of Growth Factor Receptor Signaling in Radiation Oncology

Shyhmin Huang, H. Peter Rodemann and Paul M. Harari

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## Abstract

Ionizing radiation has been shown to activate and interact with multiple growth factor receptor pathways that can influence tumor response to therapy. Among these receptor interactions, the epidermal growth factor receptor (EGFR) has been the most extensively studied with mature clinical applications during the last decade. The combination of radiation and EGFR-targeting agents using either monoclonal antibody (mAb) or small-molecule tyrosine kinase inhibitor (TKI) offers a promising approach to improve tumor control compared to radiation alone. Several underlying mechanisms have been identified that contribute to improved anti-tumor capacity after combined treatment. These include effects on cell cycle distribution, apoptosis, tumor cell repopulation, DNA damage/repair, and impact on tumor vasculature. However, as with virtually all cancer drugs, patients who initially respond to EGFR-targeted agents may eventually develop resistance and manifest cancer progression. Several

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potential mechanisms of resistance have been identified including mutations in EGFR and downstream signaling molecules, and activation of alternative member-bound tyrosine kinase receptors that bypass the inhibition of EGFR signaling. Several strategies to overcome the resistance are currently being explored in preclinical and clinical models, including agents that target the EGFR T790 M resistance mutation or target multiple EGFR family members, as well as agents that target other receptor tyrosine kinase and downstream signaling sites. In this chapter, we focus primarily on the interaction of radiation with anti-EGFR therapies to summarize this promising approach and highlight newly developing opportunities.

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**Keywords**

Radiation · Molecular targeting · Growth factor receptors

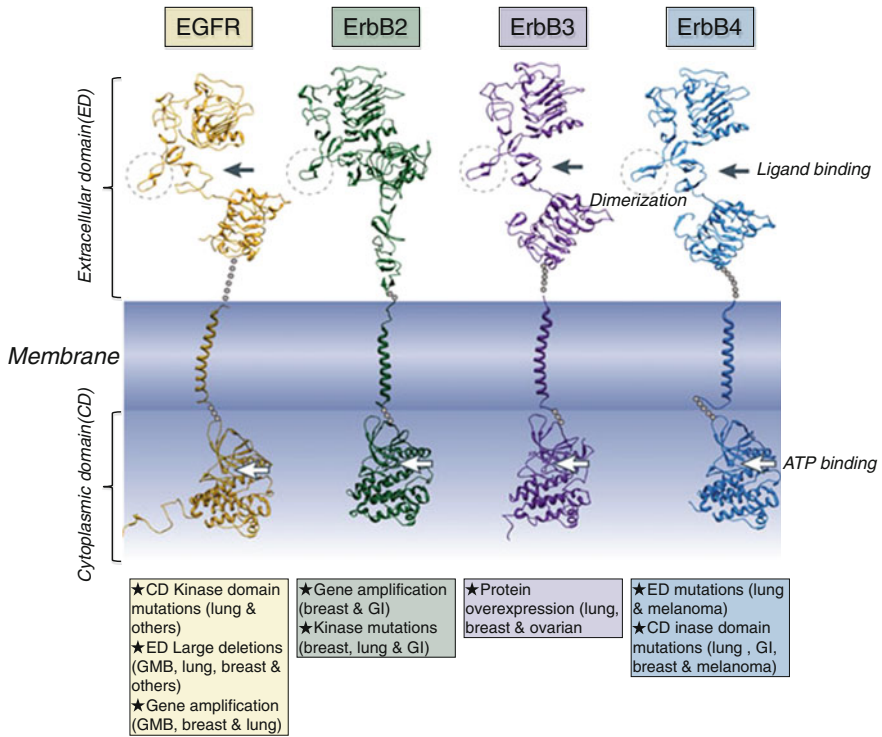
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## **1 Interaction of Radiation and Growth Factor Receptors**

Clinically relevant doses of ionizing radiation (2 Gy) are able to induce growth factor receptor signaling and ligand binding. Although precise mechanisms of radiation-induced receptor activation and stimulation of receptor-dependent downstream signaling are not fully understood, it is known that downstream pathways of stimulated receptors can mediate specific cellular radiation responses. For growth factor receptors such as the epidermal growth factor receptor (EGFR) family, preclinical data indicate a critical role of these receptors to influence intrinsic radiation sensitivity of tumor cells, as well as the DNA damage response (DDR) (Akimoto et al. 1999; Dent et al. 1999). In this chapter, specific aspects of the relevant EGFR-signaling events mediating these various radiation responses are discussed.

### **1.1 Epidermal Growth Factor Receptors**

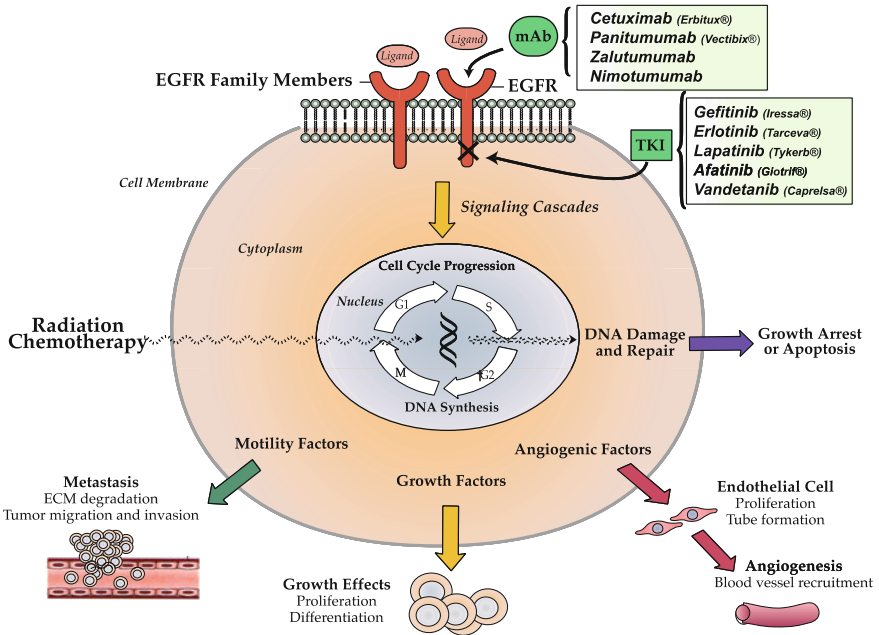
The EGFR/ErbB family of membrane-bound growth factor receptors is currently the most mature with regard to knowledge about radiation interaction and clinical trial development. This family consists of four distinct receptor isoforms, namely EGFR (ErbB1), ErbB2 (Neu, HER2), ErbB3 (HER3), and ErbB4 (Yarden and Pines 2012). These receptor molecules are transmembrane glycoproteins comprised of an extracellular ligand-binding domain and an intracellular tyrosine kinase domain (Fig. 1). Binding of the corresponding growth factors to the extracellular ligand-binding domain results in the dimerization of receptor monomers in the cell membrane. Upon dimerization, activation of the tyrosine kinase of the receptor occurs, which can stimulate various downstream signaling cascades, such as the



**Fig. 1** EGFR/ErbB family members. The four EGFR/ErbB family members are represented by the crystal structures of their three major domains, the extracellular ligand-binding domain (ED), the transmembrane domain, and cytoplasmic kinase domain (CD). The ligand-binding clefts are marked by *black arrows* and the dimerization loops by *dashed circles*. ErbB2 harbors no ligand-binding cleft, but its dimerization loop is nevertheless extended. *White arrows* mark the ATP-binding clefts. The aberrations of EGFR family members in a variety of tumor types are shown in the *bottom*. Adapted with the permission from Yarden and Pines (2012)

Ras/Raf/MEK/ERK, PKC, STAT, and PI3 K/Akt pathways (Rodemann and Blaese 2007; Rodemann et al. 2007). It is well known that these cascades are involved in the regulation of various processes controlling stress sensitivity and survival, cell cycle progression and proliferation, differentiation and apoptosis, cell matrix interactions and cell motility, and transformation as well as oncogenesis and metastasis (Fig. 2).

Nearly 50 % of all human tumors present an overexpression of EGFR or a mutational activation of this receptor (Fig. 1). These receptor alterations play an important role in the treatment response of corresponding tumor cells. In many studies, it has been demonstrated that overexpression or mutational activation of EGFR is a major mechanism leading to resistance of tumor cells against both chemo- and radiotherapy, thereby contributing to poor prognosis (Ang et al. 2002; Krause et al. 2007; Das et al. 2007; Nakamura 2007; Gupta and Raina 2010). In

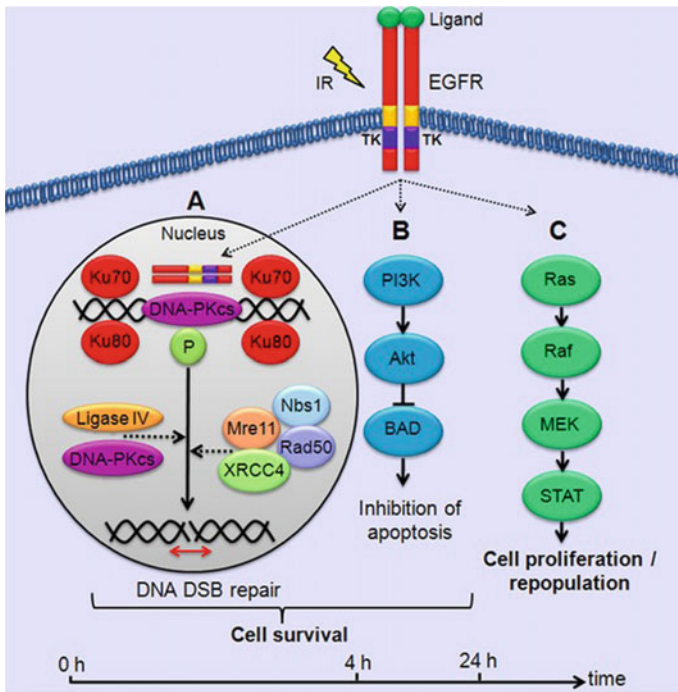


**Fig. 2** Potential mechanisms of action for combined anti-EGFR therapy with radiation. Simplified schematic illustration of the EGFR pathway and potential downstream effects of EGFR-signaling inhibition combined with radiation [copied with the permission from Harari et al. (2007)]

addition, it has been well demonstrated that EGFR activity is inducible by ionizing radiation even in the absence of ligand binding (Dent et al. 2003; Yacoub et al. 2006). EGFR and several of the downstream signaling cascades are therefore the major components of a network of cellular radiation response pathways. Exposure of cells to ionizing radiation leads to an autophosphorylation and activation of the tyrosine kinase domain of EGFR, which subsequently stimulates downstream signaling cascades (Yacoub et al. 2001, 2006; Rodemann and Blaese 2007; Toulany et al. 2007; Toulany and Rodemann 2013). Both ligand and radiation-induced EGFR signaling are primarily involved in the control of DNA damage repair, cell proliferation, and survival as well as apoptosis as shown in Fig. 3. Mainly, the PI3 K-Akt and Ras-ERK pathways have been shown to be involved in resistance to chemo- and radiotherapy due to their regulatory role in stimulating cell survival by inhibiting apoptosis (PI3 K-AKT) and promoting cell proliferation (Ras-ERK) (Schmidt-Ullrich et al. 1997; Xing and Orsulic 2005; Kim et al. 2006; Tokunaga et al. 2006; Kriegs et al. 2010; Toulany et al. 2012).

DNA double-strand breaks (DSBs) are the most lethal of the various types of DNA damage (e.g., DNA single-strand breaks, base damages, and protein-DNA cross-links), induced by ionizing radiation (IR) as well as other cellular stressors, such as many chemotherapeutic agents or endogenously generated reactive oxygen





**Fig. 3** Radiation induces EGFR activation and downstream signaling. A radioprotective role of EGFR is activated and separated into three phases as a function of time. **a** The early phase EGFR is translocated into the nucleus and activates repair signaling via binding with DNA-PK, Ku70, and Ku80 to control the rejoining of DNA double-strand breaks. **b** Activation of EGFR promotes PI3 K and Akt activation that results in the inhibition of radiation-induced apoptosis. **c** EGFR activates the Ras/Raf/MEK/ERK and STAT pathways that promote cell survival. Copied with the permission from Lilleby et al. (2011)

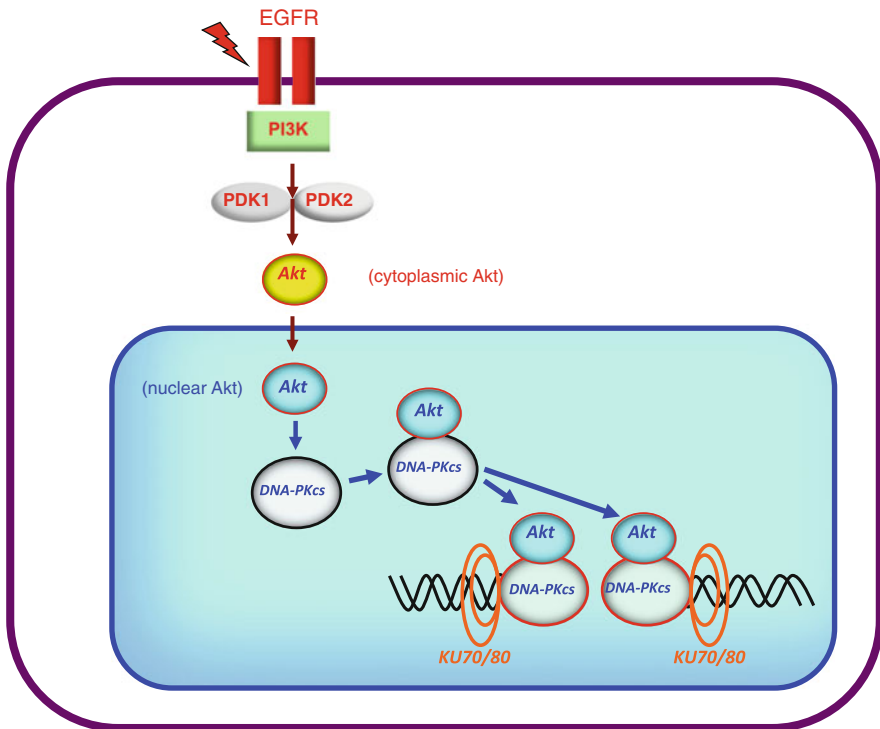
species. Additionally, DNA DSBs are produced during S-phase when the DNA replication fork encounters, for example, DNA single-strand breaks. For accurate repair of DNA DSB, the damage must be sensed and recognized by specific proteins. Moreover, appropriate signaling to the cell cycle checkpoint control system enables recruitment of the DNA-repair machinery to sites of DSBs (Scott and Pandita 2006; Rodemann et al. 2007). Depending upon the cell cycle phase in which the DNA DSBs are initiated, the cell may select between two pathways of repair, namely non-homologous end joining (NHEJ) active predominantly not only in the G1-phase, but also in the S-phase and G2-phase and the homologous recombination (HR) active in late S-phase and G2-phase only. The key enzyme of the NHEJ mechanism is the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), which for full activity forms a complex with Ku70/80 proteins that bind to the open 3'- and 5'-ends of the broken DNA double strand. Activated DNA-PK then recruits a variety of repair proteins, such as artemis, XRCC4, LIG4, and DNA-polymerase, to execute repair. In contrast, the HR repair pathway rejoins

DNA DSBs using a sister homolog as a template and, thus, facilitates a high-fidelity repair process. In concert with proteins BRCA2 and RAD54, the major player in HR is RAD51, which promotes repair of the homologous DNA double strand by HR (Shrivastav et al. 2008).

In mammalian cells, DNA DSBs are primarily repaired via the fast-responding NHEJ mechanism operating predominantly in G1-phase, for which DNA-PK is essential (Kasten et al. 1999; Kasten-Pisula et al. 2005; Wang et al. 2006). Even a small decrease in DNA-PK activity can lead to a reduced repair efficacy leading to a substantial increase in cellular radiation sensitivity. Normally, the vast majority of DNA-PK as well as Ku70/Ku80 are located within the nucleus. Interestingly, increasing evidence suggests a novel radioprotective mechanism for EGFR via a physical interaction between the EGFR protein and the DNA-PK. Based on confocal imaging, Bandyopadhyay et al. note a colocalization of DNA-PK and EGFR in the cytoplasm (Bandyopadhyay et al. 1998). In several follow-up studies, Dittmann et al. (2005a, 2008, 2009) report that ionizing radiation triggers the translocation of EGFR from the cell membrane into the nucleus in a Src-kinase-dependent manner. In the nucleus, EGFR is able to interact with DNA-PK and this interaction correlates with radiation-induced DNA-PK activity. Pretreatment with cetuximab prior to irradiation downregulates the nuclear import of and results in a decreased DNA DSB repair efficacy and cellular radiosensitization (Dittmann et al. 2005b). Current studies regarding the role of nuclear EGFR in mediating DNA DSB repair imply additional functions of nuclear EGFR in facilitating access of repair proteins to the sites of DNA DSB through opening and reorganizing heterochromatin structures (Dittmann et al. 2011).

Various molecular strategies to inhibit Akt (i.e., via siRNA and inhibitor approaches) provide strong evidence that Akt activity is necessary to stimulate efficient DNA DSB repair (Toulany et al. 2006, 2008, 2012; Toulany and Rode-mann 2010; Kang et al. 2012; Golding et al. 2009). Thus, EGFR-activated PI3 K-Akt signaling also plays a prominent role in the control and stimulation of radiation-induced DNA DSB repair. Although the detailed mechanism of how Akt is involved in the regulation of NHEJ-repair is still under investigation, recent reports demonstrate that Akt, especially the isoform Akt1, directly interacts with DNA-PK through its C-terminal domain by forming a functional complex after radiation exposure (Bozucic et al. 2008; Park et al. 2009; Toulany et al. 2012). As a consequence of this complex formation, Akt1 promotes DNA-PK accumulation at the DNA DSB site and stimulates DNA-PK kinase activity in a PI3 K-dependent manner (Toulany et al. 2012) (Fig. 4). Moreover, Akt1-dependent DNA-PK kinase activity results in DNA-PK autophosphorylation at S2056, which is essential for efficient repair (Chen et al. 2007) as well as the release of DNA-PK from the damage site (Toulany et al. 2012). Thus, Akt1 seems to be necessary for initiation, progression, and termination of the DNA-PK-dependent NHEJ-repair of DNA DSBs.

An alternative or concomitant pathway regulating DNA DSB repair via Akt is the upregulation of MRE11 expression after Akt activation through GSK3 $\beta$ / $\beta$ -catenin/LEF-1 pathway (Bouchaert et al. 2012; Fraser et al. 2011).



**Fig. 4** PI3 K-dependent Akt regulation of DNA DSB. Akt activated via erbB-PI3 K signaling forms a complex with DNA-PKcs in the nucleus to stimulate initiation, progression, and termination of DNA DSB repair through the NHEJ mechanism

MRE11 is a central protein, which after radiation exposure of cells binds to the proteins RAD50 and NBS1 to form the MRN complex. This complex rapidly accumulates to damage site and appears to be the major sensor of DNA DSBs. At the DNA DSB site, MRN subsequently recruits, together with other proteins, the signaling protein ATM, which mediates cell cycle checkpoint control to allow DNA repair (Lavin 2007). Approximately, 85 % of DNA DSBs induced by ionizing radiation are repaired within the first 2–3 h post-irradiation via the so-called fast component of DNA DSB repair, which is independent of ATM function (Jeggio et al. 2011). The remaining 15 % of DNA DSBs mainly composed of complex lesions are repaired in an ATM-dependent manner via the so-called slow component (Goodarzi et al. 2010; Beucher et al. 2009). Inhibition of Akt leads to a downregulation of MRE11 protein; thus, Akt1 may function in the control of DNA repair additionally as a regulator of the slow components of DNA repair. This potential function would be complementary to the role of Akt in the DNA-PK-dependent fast repair process.

Thus, the importance of both EGFR signaling via signaling cascades and the nuclear translocation pathway of EGFR for the efficacy of DNA DSB repair

through the DNA-PKcs-dependent NHEJ-repair mechanism underscores the efficacy of targeting the EGFR as well as the PI3 K-Akt pathway to improve outcome with radiation therapy.

## 1.2 Insulin Growth Factor Receptor 1

The insulin-like growth factor receptor 1 (IGF-1R) is another major growth factor receptor of relevance for mediating the radiation response of tumor cells (Larsson et al. 2005; Miller and Yee 2005; Riesterer et al. 2011). IGF-1R is a hetero-tetrameric protein with 2 identical  $\alpha$ -subunits presenting the extracellular IGF-binding site. The intracellular domain of the two transmembrane  $\beta$ -subunits contains the tyrosine kinase activity responsible for transmitting the signal to intracellular cascades. Similar to the activation of ErbB receptors, binding of the specific ligand IGF results in conformational changes of the IGF-1R, autophosphorylation of specific tyrosine residues, and activation of downstream intracellular signaling cascades (Riesterer et al. 2011). As with EGFR, the Ras-Raf-MAPK and PI3 K-Akt cascades represent major pathways activated by IGF-1R resulting in the activation of a variety of cellular responses, such as survival, proliferation, differentiation, adhesion, and motility (Riesterer et al. 2011). The important function of IGF-1R as cell survival factor has been shown in a variety of cell types. Moreover, IGF-1R reduces the inducibility of apoptosis. Several lines of evidence suggest IGF-1R as a mediator of treatment resistance to cytotoxic agents and ionizing radiation (Macaulay et al. 2001; Peretz et al. 2001; Rochester et al. 2005). Downregulation of IGF-1R by antisense RNA impairs activation of the DNA damage sensor protein ATM kinase and can thus enhance cellular radiation sensitivity (Macaulay et al. 2001). In addition, several anti-IGF-1R antibodies including cixutumumab show to augment radiation response in a variety of pre-clinical models (Allen et al. 2007). Mechanistically, IGF-1R inhibition has been found to promote apoptosis and inhibit repair of radiation-induced DNA damage through interference with the expression of DSB repairing Ku-proteins and their binding to DNA (Cosaceanu et al. 2007).

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## 2 EGFR-Targeting Agents

Despite gradual advancement in concurrent radiochemotherapy treatment regimens, the overall toxicity of treatment and relative lack of specificity for individual patients can limit the ultimate clinical effectiveness. These challenges have stimulated the development of molecular-targeted therapies that may provide more specific attack on tumor cells with less collateral impact on surrounding normal tissues. There are a rapidly expanding number of molecular-targeting agents against various growth factor receptors under development and in current clinical use. By targeting aberrant expression and/or mutations in growth factor receptor signaling pathways that are more prevalent in cancer cells, molecular-targeting agents offer

the potential to improve therapeutic outcome. The EGFR-targeting agents have been a focus of research during the last two decades. Following the early work by Mendelsohn and colleagues (Mendelsohn 2003; Masui et al. 1984) who demonstrated the anti-tumor effect of a monoclonal antibody (mAb) to EGFR, two distinct classes of EGFR inhibitors, mAb and small-molecule tyrosine kinase inhibitor (TKI) against EGFR, have demonstrated preclinical and clinical promise and gained US Federal Drug Administration (FDA) approval in last 10 years (Table 1) (Harari and Huang 2004; Dassonville et al. 2007; Yarden and Pines 2012). Although both approaches can block EGFR activation, the mode of action and pharmacokinetic profile of mAbs and TKIs vary considerably (Table 2). Anti-EGFR mAbs block

**Table 1** EGFR-targeting agents

Name	Manufacturer	Molecule	Specificity	Development status
Cetuximab <i>Erbbitux®</i> C225	ImClone systems Inc. Bristol-Myers Squibb	Mouse-human Chimeric IgG1	EGFR	FDA approved for CRC patients with wt KRAS Approved for use in combination with radiotherapy in HNSCC patients
Panitumumab <i>Vectibix®</i> <i>ABX-EGF</i>	Amgen Abgenix	Human IgG2	EGFR	Approved for CRC patients with wt KRAS
Zalutumumab HuMax-EGFr	Genmab	Humab IgG1	EGFR	Approved for Fast Track by FDA in HNSCC patients who have failed standard therapies
Nimotuzumab <i>h-R3</i> <i>TheraCIM</i>	YM BioScience	Human IgG1	EGFR	Approved in Asia and Europe. In the USA, in late-stage development and testing for FDA review
Sym004	Symphogen A/S	A mixture of two chimeric IgG1 s	EGFR	Phases I and II in HNSCC and CRC
MEHD7945A	Genentech	Human bi-specific IgG1	EGFR HER3	Phases I and II in HNSCC and CRC
Gefitinib <i>Iressa®</i> ZD1839	AstraZeneca	Reversible TKI	EGFR	Approved for patients with advanced NSCLC
Erlotinib <i>Tarceva®</i> <i>OSI-774</i>	Genentech	Reversible TKI	EGFR	Approved for patients with advanced NSCLC patients Approved for use in combination with gemcitabine in patients with advanced pancreatic cancer
Vandetanib <i>Caprelsa®</i> ZD6474	AstraZeneca	Reversible TKI	EGFR VEGFR	Approved for patients with late-stage thyroid cancer

(continued)

**Table 1** (continued)

Name	Manufacturer	Molecule	Specificity	Development status
Lapatinib <i>Tykerb</i> ® GW572016	GlaxoSmithKline	Reversible TKI	EGFR HER2	Approved for use in combination with capecitabine in patients with breast cancer
Afatinib <i>Giotrif</i> ® BIBW2992	Boehringer Ingelheim	Irreversible TKI	EGFR HER2	Phase III in breast and NSCLC Phase II in prostate and HNSCC
Neratinib HKI-272	Puma biotechnology	Irreversible TKI	EGFR HER2	Phases II and III in breast, brain and NSCLC patients
Dacomitinib PF-00299804	Pfizer	Irreversible TKI	EGFR HER2 HER3 HER4	Phase II & III in HNSCC & NSCLC patients Phase II in patients with recurrent glioblastoma or advanced gastric cancer

*Ab* Antibody; *CRC* Colorectal cancer; *FDA* Food and drug administration; *HNSCC* Head and neck squamous cell carcinoma; *NSCLC* Non-small cell lung cancer; *TKI* Tyrosine kinase inhibitor

**Table 2** mAb versus TKI

Parameter	mAb	TKI
Specificity	+++	+
Off-target activity	–	+
Size (kDa)	>150	<1
Administration	i.v (weekly)	Oral (daily)
Half-life	>4 days	<1 day
Receptor internalization	+	–
Block lateral receptor signaling	+	–
Immune response (ADCC, CDC)	+	–
Consistent pharmacokinetics	+	–
Nausea/Diarrhea	+	++
Acne-like rash	+++	++

*ADCC* Antibody-dependent cell-mediated cytotoxicity

*CDC* Complement-dependent cytotoxicity

ligand binding in the extracellular domain and TKIs directly inhibit the activation of cytosolic catalytic domain of the EGFR. Anti-EGFR mAbs are delivered intravenously since they are large molecules that are susceptible to degradation in GI tract and have a long half-life. There also exists the potential for stimulation of immunological responses with the use of mAbs. On the contrary, small-molecule TKIs are administrated orally due to their short half-life and effective absorption across the GI tract.

## 2.1 Anti-EGFR Antibody

A series of anti-EGFR mAbs have been used in the treatment of a variety of cancers as shown in Table 1. These antibodies have some potentially important differences in their structure and targeted EGFR epitope that may influence efficacy and toxicity. Among these, cetuximab (Erbix<sup>®</sup>) and panitumumab (Vectibix<sup>®</sup>) are the most well-studied anti-EGFR mAbs with regard to mature clinical data. Cetuximab is a chimeric mAb with 65 % of human and 35 % of murine composition that received FDA approval in 2004 for the treatment of irinotecan-refractory colorectal cancer. Cetuximab is also the first EGFR-targeting agent approved to combine with radiotherapy in the treatment of HNSCC patients based on promising results from preclinical studies and phase III clinical trials (Nyati et al. 2006; Harari and Huang 2006; Bonner et al. 2006, 2010). The remaining portion of mouse immunoglobulin (IgG) in cetuximab might account for the low rate of infusional allergic reactions that have been described in the clinic with this drug. By contrast, panitumumab which is a fully human antibody may be less likely to elicit such host allergic responses. In addition, cetuximab may exhibit a potential therapeutic benefit to enhance antibody-dependent cellular cytotoxicity (ADCC) due to its IgG1 framework (Pahl et al. 2012; Kurai et al. 2007). Panitumumab, constructed on an IgG2 framework, does not possess this immune functionality. Ultimately, the comparative therapeutic efficacies of these two FDA-approved distinct anti-EGFR mAbs will be best evaluated in the context of controlled clinical trials.

## 2.2 EGFR Tyrosine Kinase Inhibitor

A second approach to disrupt EGFR function involves the use of synthetic quinazoline-derived TKIs that bind to the ATP-binding pocket of the EGFR tyrosine kinase domain and subsequently inhibit receptor activation. Gefitinib (Iressa<sup>®</sup>) and erlotinib (Tarceva<sup>®</sup>) are the first two EGFR-specific TKI to gain FDA approval for use in the treatment of chemotherapy-refractory NSCLC and advanced pancreatic cancer patients who have not received previous chemotherapy. Other FDA-approved drugs, such as vandetanib (Caprelsa<sup>®</sup>) and lapatinib (Tykerb<sup>®</sup>), inhibit multiple receptors in addition to EGFR, such as VEGFR and HER2. There are also several other promising multitargeted TKIs currently in the clinical trial seeking FDA approval such as afatinib, neratinib, and dacomitinib (Table 1).

To achieve a maximal therapeutic effect, the combination of an anti-EGFR mAb and a TKI has been explored as a potential cancer treatment strategy. Although these two classes of agents both target EGFR, they differ in their mode of action and carry distinct toxicity profiles as described above (Dassonville et al. 2007). By microarray analysis, Baselga and colleagues identified 45 genes that are differentially expressed after treatment with cetuximab versus gefitinib, including genes related to cellular proliferation and differentiation, DNA synthesis and repair, and angiogenesis and metastasis (Matar et al. 2004). Although the clinical significance

of the differences in activity between anti-EGFR mAb and TKI is not yet clear, preclinical studies have demonstrated a synergistic effect in several xenograft model systems when cetuximab is administered in combination with gefitinib or erlotinib compared with single agent treatment (Huang et al. 2004; Matar et al. 2004; Jimeno et al. 2005). These two agents given together are able to exert a superior induction in apoptosis and blockade of EGFR activation and downstream signaling. Interestingly, it is further shown that EGFR TKI can overcome acquired resistance to anti-EGFR mAb in cell culture and animal models (Huang et al. 2004; Regales et al. 2009; Brand et al. 2011). A phase I clinical trial confirms that treatments with cetuximab and gefitinib are feasible in patients with recurrent NSCLC and warrant further clinical investigation (Ramalingam et al. 2008).

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### **3 Combination of EGFR-Targeting Agents with Radiation**

#### **3.1 Preclinical Studies—Biological Mechanisms of Action**

A series of preclinical studies have provided proof-of-principle that blockade of EGFR can enhance radiation toxicity in a variety of tumor model systems (Krause et al. 2006; Baumann et al. 2007). Early studies identified the capacity of cetuximab to increase radiosensitivity in vitro and augment the effect of single dose and fractionated radiations in vivo (Huang et al. 1999; Milas et al. 2000). In addition, a more clinically relevant end point, improved local tumor control after single dose of irradiation in combination with cetuximab, was also reported in an animal model (Nasu et al. 2001). Thereafter, a series of anti-EGFR mAbs and TKIs were shown to augment radiation response in a variety of tumor models, including gefitinib, erlotinib, and panitumumab (Huang et al. 2002; Bianco et al. 2002; Chinnaiyan et al. 2005; Kruser et al. 2008). The potential underlying mechanisms for the radiosensitizing effect of EGFR inhibitors include radiation-induced repopulation, cell cycle arrest, senescence, apoptosis, DNA damage repair, and angiogenesis (Fig. 2).

##### **3.1.1 Repopulation, Cell Cycle Progression, and Senescence**

The negative impact of radiation-induced repopulation of cancer cells on the outcome of fractionated irradiation has been shown in preclinical and clinical studies, particularly for HNSCC. Preclinical data suggest that radiation-induced EGFR activation results in the accelerated repopulation of surviving clonogenic tumor cells and that EGFR inhibition counteracts this mechanism of radioresistance (Schmidt-Ullrich et al. 1997; Baumann et al. 2007). Simultaneous application of cetuximab during radiation with 30 fractions over 6 weeks in FaDu cancer cells improved local tumor control via the inhibition of tumor cell repopulation when compared to radiation alone (Krause et al. 2005). Further studies suggest that perturbations in cell cycle progression provide an underlying mechanism for the



anti-proliferation and radiosensitization effects of EGFR inhibitors (Ahsan et al. 2009).

Both anti-EGFR mAbs and TKIs have been shown to alter cell cycle distribution, with a 10–20 % shift from S-phase to G0/G1 arrest (Harari and Huang 2001; Huang et al. 2002; Chinnaiyan et al. 2005). Therefore, one hypothesis is that the radiosensitization effect of EGFR inhibitors may result from a decrease of the percentage of cells in S-phase, a relatively radioresistant phase, with concomitant increase in the more radiosensitive G1-phase of the cell cycle. Furthermore, the combined effects of G1 arrest induced by EGFR inhibitors, together with G2/M arrest induced by ionizing radiation, ultimately result in cell cycle checkpoint deregulation and subsequent apoptosis. In addition, EGFR inhibitors may trigger cellular senescence, an irreversible cell cycle arrest in response to double-strand break (DSB) produced by radiation. By screening 11 NSCLC cell lines in both *in vitro* and *in vivo*, a study showed that treatment with cetuximab or erlotinib led to pronounced cellular senescence but not apoptosis following radiation in 5 of 11 cell lines (Wang et al. 2011). Furthermore, EGFR inhibitor-induced senescence was associated with increased unrepaired DSB. These results recognize EGFR inhibitor-induced senescence as a meaningful contributor to the overall loss of tumor cell viability following radiation.

### 3.1.2 Apoptosis

EGFR inhibition may sensitize cells to apoptosis induced by radiation. In many preclinical studies, potentiation of apoptosis is shown to contribute to the radiosensitizing effect of EGFR inhibitors (Huang et al. 1999, 2002; Chinnaiyan et al. 2005; Kruser et al. 2008). The loss of EGFR alone is often insufficient to induce apoptosis completely. However, downregulation of EGFR-mediated survival signals has been shown to sensitize cells to apoptosis (Goel et al. 2007). EGFR inhibition downregulates Ras/Erk- or PI3 K/Akt-dependent survival pathways and is associated with a pro-apoptotic shift by inhibiting Bcl-2/Bcl-X<sub>L</sub> and/or upregulating Bax/Bad (Gilmore et al. 2002; Sheng et al. 2007; Kruser et al. 2008). In addition, studies report that gefitinib can induce apoptosis through a p53-dependent signaling pathway, and p53 mutation in combination with p21 expression in colorectal cancer can serve as a predictor of resistance to gefitinib (Ogino et al. 2005; Rho et al. 2007; Chang et al. 2008). Recently, we found a loss of p53 in EGFR inhibitor-resistant cells that associate with a resistance phenotype to not only EGFR inhibitor, but also to radiation (Huang et al. 2011). Restoration of functional p53 in resistant cells can re-establish sensitivity to EGFR inhibitor and radiation via induction of cell cycle arrest, apoptosis, and DNA damage repair. Additional studies show that p53 is involved in the regulation of EGFR downstream PI3 K/AKT and ERK pathways (Singh et al. 2002; Bouali et al. 2009; Sauer et al. 2010; Zwang et al. 2011). All these results suggest that p53 may regulate sensitivity to EGFR inhibitors and radiation by modulating EGFR downstream signaling functionality and apoptosis induction.

### 3.1.3 DNA Damage Repair

Another mechanism of synergy between EGFR inhibition and radiation is through the inhibition of DNA DSB damage repair, mainly NHEJ (Szumiel 2006; Meyn et al. 2009; Lieber 2010). As described above, EGFR is shown to translocate into the nucleus after radiation to act either as a transcription factor or as a cofactor for the DNA-PK-Ku78/80 repair complex (Szumiel 2006; Chen and Nirodi 2007). When EGFR is blocked by cetuximab or gefitinib, a substantial amount of DNA-PK is retained in the cytosol due to a stalled nuclear import of the EGFR complex (Bandyopadhyay et al. 1998; Dittmann et al. 2005b; Friedmann et al. 2006). As a consequence, radiation-induced DNA-PK activation is abolished and leads to impaired repair as shown by increased numbers of  $\gamma$ -H2AX foci and significantly reduced clonogenic survival (Dittmann et al. 2005b). Additionally, a report indicates that gefitinib suppresses DNA-repair capacity via another DNA-repair protein NBS1, not DNA-PK (Tanaka et al. 2008) in NSCLC cells. In addition, erlotinib is shown to attenuate homologous recombinational repair of DSBs in breast cancer cells (Li et al. 2008b). The relationship between the EGFR signaling and the DNA-repair process has not been fully clarified, but these observations raise the possibility that impairment of DNA-repair processes, especially for DSB repair, may be involved in the modulation of radiosensitivity by EGFR inhibitors.

### 3.1.4 Angiogenesis

It is becoming clear that EGFR inhibitors can interfere with tumor–stromal interactions, such as angiogenesis, and this anti-angiogenic effect of EGFR inhibitors is considered as a potential underlying mechanism for EGFR inhibitor-mediated radiosensitization (Guillamo et al. 2009; Nijkamp et al. 2013). Although radiation oncologists have expressed caution about the induction of a radioresistant hypoxia environment from inhibition of angiogenesis, a body of preclinical and clinical data has emerged in support of combining angiogenesis inhibitors with radiation (O'Reilly 2006; Kleibeuker et al. 2012). An alternative hypothesis suggests that anti-angiogenic agents may serve to transiently “normalize” the abnormal structure and function of tumor vasculature to make it more efficient for oxygen and drug delivery (Jain 2005; Ma and Waxman 2008; Fukumura and Jain 2007). In addition, by determining the optimal scheduling and dose of anti-angiogenic agents, the combination of radiation with anti-angiogenic agents may improve therapeutic outcome via vessel normalization (Dings et al. 2007). Therefore, detailed investigation regarding the temporal changes of the tumor microenvironment appears important for our ultimate understanding of EGFR/radiation interactions.

Cetuximab or gefitinib have been extensively reported to suppress neovascularization in a variety of tumor xenografts (Huang and Harari 2000; Huang et al. 2001; She et al. 2003). The anti-angiogenic mechanism of EGFR inhibitors involves not only suppression of several angiogenic factors that are produced by tumor cells, but also direct inhibition of the proliferation and migration of endothelial cells, endothelial-associated pericytes, and perivascular cells (Al-Nedawi et al. 2009; Iivanainen et al. 2009). For example, simultaneous

application of cetuximab during fractionated irradiation is found to improve reoxygenation of tumor xenografts that contributes to improved local control of FaDu tumors (Petersen et al. 2003; Krause et al. 2005). Further studies indicate that treatments with EGFR inhibitors result in a reduced expression of the hypoxia-related protein and hypoxia-inducible factor alpha (HIF-1 alpha) (Riesterer et al. 2009; Pore et al. 2006). Consistently, several studies confirm that EGFR inhibitors can modulate the microenvironment by vascular normalization to improve radiotherapy efficacy (Gan et al. 2009; Cerniglia et al. 2009; Qayum et al. 2009). Imaging studies further identify changes in vessel morphology in erlotinib-treated tumor xenografts accompanied by increased tumor blood flow, decreased vessel permeability, and reduced hypoxia that are typical characteristics of tumor vascular normalization. These changes correlate with improved drug delivery and increased response to radiation (Cerniglia et al. 2009). These findings suggest that improvement in blood flow leads to better drug delivery or increased tumor oxygenation and thereby offer a rationale for why EGFR inhibitors may enhance the radiation response of tumors.

## 3.2 Clinical Studies

### 3.2.1 Cetuximab + Radiotherapy

During the last 10 years, the combination of EGFR inhibitors with radiation has been actively tested in patients with a variety of tumors including HNSCC, NSCLC, colon, esophageal, breast, and brain (Al-Ejeh et al. 2013; Welsh et al. 2013). Results from phases I and II clinical trials indicate that combining EGFR inhibitors with radiotherapy or chemoradiotherapy is generally well tolerated with minimal overlapping toxicity (Table 3). In 2006, the first landmark multicenter phase III clinical trial was reported demonstrating that the administration of cetuximab to radiotherapy can provide a survival advantage for HNSCC patients. Bonner et al. compared the efficacy of radiotherapy alone to radiotherapy plus cetuximab in 424 patients with locoregionally advanced HNSCC. The benefit of the association of cetuximab with radiotherapy as compared to radiotherapy alone was demonstrated in terms of locoregional control (median duration: 24.4 vs. 14.9 months,  $p = 0.005$ ), progression-free survival (3-year rate: 42 vs. 31 %,  $p = 0.04$ ), and overall survival (median duration: 49 vs. 29.3 months,  $p = 0.03$ ) (Bonner et al. 2006). Recently, the five-year follow-up report confirms stability of the advantage for the cetuximab arm with a 10 % improvement (45.6 vs. 36.4 %,  $p = 0.018$ ) in overall survival compared to the radiation alone arm (Fig. 5) (Bonner et al. 2010). With the exception of the characteristic cutaneous toxicity of cetuximab (acneiform rash), the incidence of grade 3 toxicities was not significantly higher in the group treated with cetuximab. Moreover, the authors showed that for the patients treated with cetuximab, overall survival was significantly improved in those who experienced an acneiform rash of at least grade 2 severity compared with patients with no rash or grade. Importantly, this is the first trial showing that the outcomes of radiotherapy can be significantly improved by the addition of a

**Table 3** Selected clinical trials combining EGFR inhibitors with radiation

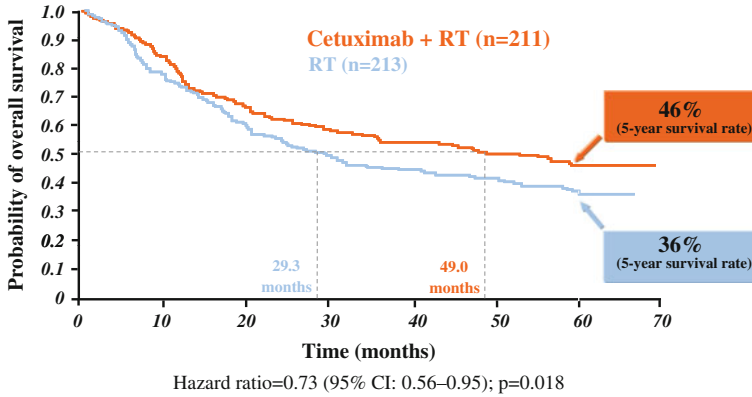
Treatment	Phase	Cancer (N)	Response	Survival index	Reference
RT versus RT + cetuximab	III	H&N (424)	LRC: 14.9 versus 24.4 months OR: 64 versus 74 %	OS at 5 years: 29.3 versus 49 months	Bonner et al. (2006, 2010)
Platinum-based CRT versus platinum-based CRT + cetuximab	III (RTOG0522)	H&N (895)		OS at 2.5 years: 79.7 versus 82.6 months PFS: 64.3 versus 63.4 months	Ang et al. (2011)
Induction chemotherapy followed by CRT and cetuximab during sequential treatment	II	H&N (39)		OS at 3 years: 74 % PFS: 73 %	Argiris et al. (2010)
RT, cisplatin, 5-FU, and cetuximab (rapid alternating treatment)	II	H&N (45)	OR: 91 % CR: 71 %	OS at 3.5 years: 32.6 months PFS: 21 months	Merlano et al. (2011)
IMRT + cetuximab		NSCLC (49)	OR: 63 %	Median OS: 19.5 months Median PFS: 10.9 months	Jensen et al. (2011)
RT, carboplatin, pemetrexed versus RT, carboplatin, pemetrexed + cetuximab	II	NSCLC (101)		OS at 1.5 years: 58 versus 54 %	Govindan et al. (2011)
RT, CRT, and cetuximab	II	Esophagus (60)	CR:70 %		Safran et al. (2008)
RT, gemcitabine, oxiliplatin, and cetuximab	II	Pancreas (69)		Median OS: 19.2 months	Crane et al. (2011)
CRT, bevacizumab, erlotinib	II	H&N (48)	OR:77 %	OS at 3 years: 82 % PFS: 71 %	Hainsworth et al. (2011)
RT, cisplatin, erlotinib	I/II	H&N (31)	CR:74.2 %		Herchenhorn et al. (2010)
RT versus RT + erlotinib	II	NSCLC (23)	OR: 55.5 versus 83.3 %		Martinez et al. (2008)

(continued)

**Table 3** (continued)

Treatment	Phase	Cancer (N)	Response	Survival index	Reference
RT, temozolomide, erlotinib	II	GBM (65)		Median OS: 19.3 months	Prados et al. (2009)
CRT, erlotinib	II	Esophagus (24)	CR:46 %		Li et al. (2010)
RT, 5-FU, hydroxyurea, gefitinib	II	H&N (69)	CR:90 %	OS at 4 years: 74 % PFS: 72 %	Cohen et al. (2010)
RT, gefitinib	II	H&N (23)		OS at 2 years: 72.1 % PFS: 63.6 %	Lewis et al. (2012)
RT + gefitinib	II (RTOG0211)	GBM (147)		Median OS:11.5 months DMC:32 versus 40 %	Chakravarti et al. (2013)
CRT versus CRT + gefitinib	II	Esophagus (80)		OS at 3 years: 28 versus 42 %	Rodriguez et al. (2010)

RT Radiotherapy; CRT Chemoradiation; N Number of patients; LRC Local regional control; OR Overall response; CR Complete response; OS Overall survival; PFS Progression-free survival; IMRT Intensity-modulated radiation therapy  
DMC Distant metastatic control



**Fig. 5** Survival advantage of cetuximab in combination with radiotherapy from phase III clinical trial in HNSCC patients. Adapted with the permission from Bonner et al. (2010)

molecular-targeting agent without significantly increasing the radiation toxicity. Overall, results from this trial validate the preclinical models that predicted a significant improvement of tumor control based on the radiosensitizing and apoptosis-promoting effects of cetuximab (Huang et al. 1999).

Although the addition of cetuximab to radiotherapy improves locoregional control and survival in HNSCC, the addition of cetuximab to platinum-based chemoradiotherapy did not lead to an improved outcome based on the recent phase III trial, RTOG 0522 (Table 3) (Ang et al. 2011). This study evaluates the addition of cetuximab to a standard-of-care concurrent radiation/cisplatin regimen in 895 patients with stage III or IV nonmetastatic HNSCC. Following a median 2.4 years of follow-up, the initial results show that the addition of cetuximab to the radiation/cisplatin regimen did not improve progression-free (64.3 % without cetuximab and 63.4 % with cetuximab) or overall survival (79.7 vs. 82.6 %). The addition of cetuximab was associated with higher rates of mucositis and cetuximab-induced skin reactions. Further analysis is underway to determine whether tumor human papillomavirus status affects the relative efficacy of the chemoradiotherapy plus cetuximab regimen.

There are a number of clinical trials evaluating the safety and efficacy of cetuximab and other anti-EGFR agents in association with chemoradiotherapy, either as primary or as postoperative treatment in a spectrum of cancer patients (Table 3). For example, Argiris et al. conducted a trial of 39 patients with advanced HNSCC treated with induction docetaxel, cisplatin, and cetuximab followed by concurrent cisplatin and cetuximab with 70 Gy radiation. Preliminary reports from this study demonstrate that cetuximab-containing regimen results in excellent long-term survival and safety (Argiris et al. 2010). The progression-free survival and overall survival were 70 and 74 %, respectively. While most radiotherapy clinical trials were generally still using 2D techniques, one recent phase II study (NEAR trial) in NSCLC patients combined cetuximab with a 4D

intensity-modulated radiation treatment (IMRT) that permits dose escalation while relatively sparing surrounding normal tissues (Jensen et al. 2011). The study showed encouraging preliminary results for safety, local regional control, and survival rate in patients treated with IMRT and cetuximab. Several other phase II studies also showed the efficacy and safety of the addition of cetuximab to chemoradiotherapy in other tumor sites such as esophageal and pancreatic cancer (Crane et al. 2011; Safran et al. 2008).

### 3.2.2 TKI + Radiotherapy

Beyond cetuximab, EGFR TKIs have also been evaluated in combination with radiotherapy in a number of cancer types (Table 3). Notable among these is a phase II study evaluating erlotinib plus temozolomide during and after radiation therapy in patients with glioblastoma multiforme or gliosarcoma (Prados et al. 2009). In addition to 75 mg/day of temozolomide, patients were given 100 mg/day of erlotinib and during radiation and were escalated after radiation therapy to 150 mg/day. The median survival was 5.2 months longer than that projected in comparison with historical controls patients without erlotinib. Interestingly, it was found that patients with methylguanine methyltransferase (MGMT) methylation had a better overall survival compared to patients without MGMT methylation (25.5 vs. 14.6 months). These findings provide information regarding patient selection for future clinical trials, though further studies are necessary to determine the importance of MGMT methylation in this patient cohort.

Several phase II trials have evaluated the combination of erlotinib and radiotherapy in the definitive treatment of HNSCC and NSCLC, although published data are limited (Mehta 2012). For example, a study evaluated the combination of erlotinib with a standard cisplatin-based chemoradiotherapy regimen in patients with stages III and IV HNSCC (Herchenhorn et al. 2010). At a median follow-up of 37 months, it was found that 3-year progression-free survival and overall survival rates were 61 and 72 %, respectively. Similar to cetuximab, further analysis revealed a trend toward superior overall survival in patients who developed acneiform rash compared with those who did not have rash. Because of the high complete response rate, the authors advocated further evaluation of this treatment regimen in phase III clinical trial. In addition, a prospective phase II study found that radiation and concurrent erlotinib used in the treatment of patients with NSCLC showed promising results without an increase in toxicity (Martinez et al. 2008). Patients with unresectable stage I to IIIA NSCLC received 3D thoracic radiotherapy with or without concomitant erlotinib. The addition of erlotinib did not appear to increase radiation-associated toxicities. Erlotinib-related adverse events included mild-to-moderate skin rash (61.5 %) and diarrhea (23 %). The response rate was 55.5 % in the radiotherapy arm compared with 83.3 % in the radiotherapy plus erlotinib arm. Thus, based on the evidence, further clinical investigation into the combination of radiotherapy with EGFR TKI appears warranted.

## 4 Resistance to EGFR-Targeted Therapy

Although the first generation of phase III clinical trials combining EGFR TKI with chemotherapy did not demonstrate a survival benefit (Giaccone et al. 2004; Herbst et al. 2004, 2005), the first major phase III trial combining cetuximab with radiation confirms a strong survival advantage for HNSCC patients. While this compelling clinical result provides powerful stimulus to better understand mechanisms underlying the favorable interaction between EGFR blockade and radiation, many patients who initially respond well to treatments still manifest tumor recurrence (Jackman et al. 2010; Sequist et al. 2011b). Moreover, the addition of EGFR inhibitors to chemoradiotherapy in several clinical trials, although feasible, does not lead to a clinical benefit. Both intrinsic and acquired resistance to EGFR inhibitors are now well-recognized occurrences in clinical trials and emerge as potential treatment barriers for the optimization of EGFR-targeted therapy (Jackman et al. 2010; Wheeler et al. 2010; Sequist et al. 2011b).

### 4.1 EGFR Mutations

Somatic mutations in the EGFR play a crucial role in regulating the efficacy of EGFR inhibitors. In 2004, a series of landmark studies identified several EGFR mutations in the tyrosine kinase domain that significantly correlate with clinical responsiveness to gefitinib or erlotinib in NSCLC cancer patients (Lynch et al. 2004; Paez et al. 2004; Pao et al. 2004; Pao and Miller 2005). These mutations included in-frame deletion of amino acids 746–750 in exon 19, and a point mutation in exon 21 (L858R) that are around the ATP-binding pocket of the tyrosine kinase domain. Similarly, a recent study in HNSCC identified a somatic mutation in the ligand-binding domain (P546S) that may contribute to increased sensitivity to cetuximab (Bahassi et al. 2013). Subsequently, it was found that patients with active EGFR mutation who initially responded to gefitinib or erlotinib developed acquired resistance after a median of 10–14 months on EGFR TKI treatment (Jackman et al. 2010; Zhou et al. 2011). It is now known that appropriately 50–60 % of patients with acquired resistance to gefitinib have a second-site mutation in T790 M (Pao et al. 2005). It can also be detected as a mechanism of primary resistance in patients with no prior exposure to EGFR TKI, and its presence has been significantly associated with poorer outcome on EGFR therapy (Rosell et al. 2011). Originally, it was first predicted that the T790 M mutation sterically hindered the binding of TKIs to the ATP pocket of EGFR due to the introduction of a bulky Met residue, thus resulting in drug resistance (Kobayashi et al. 2005). However, recent biochemical and structural studies reveal that TKI resistance associated with T790 M mutation is not attributable to steric blocking of TKI binding, as previously predicted, but rather to the increased affinity for ATP that dramatically decreases the potency of the drug at cellular concentration of ATP (Yun et al. 2008; Eck and Yun 2010; Yoshikawa et al. 2013). In addition to EGFR

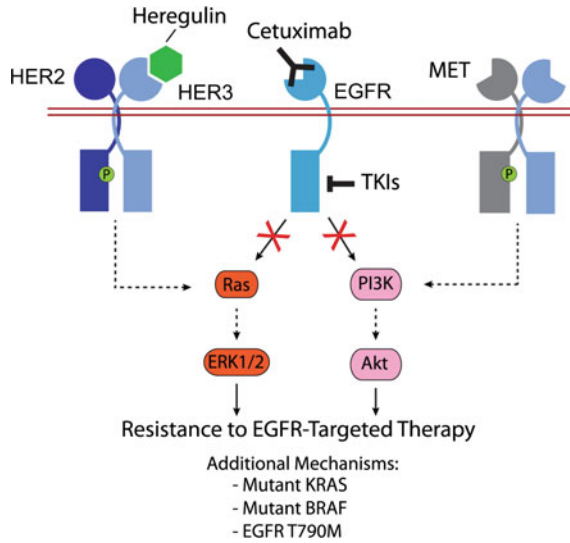


T790 M mutation, several other mutations in EGFR kinase domain have also been associated with acquired resistance to EGFR TKI including the T854A in exon 21 (Bean et al. 2008), L747S (Costa et al. 2008), and D761Y (Balak et al. 2006) in exon 19. However, the frequency of these mutations appears to be much lower than T790 M mutation.

The discovery of these EGFR-activating mutations raises important questions about the response of mutant EGFR to ionizing radiation. In contrast to the radioresistance conferred by EGFR overexpression, several *in vitro* studies showed that the NSCLC cell lines bearing activating EGFR mutations were more sensitive to radiation, evidenced by poor clonogenic survival, incomplete DSB repair, and either induction of apoptosis or development of micronuclei in response to ionizing radiation (Das et al. 2006; Sato et al. 2012). Further mechanistic studies demonstrated that unlike wt EGFR, receptors with activating mutations were defective in radiation-induced translocation to the nucleus and failed to bind DNA-PK (Das et al. 2007). Interestingly, tumor cells with gefitinib-resistant T790 M mutant still exhibited enhanced sensitivity to radiation (Das et al. 2006). Thus, despite the differences in sensitivity to gefitinib, NSCLC cells with different kind of EGFR mutations show similar responses to radiation. Although the presence of EGFR mutations in NSCLC is associated with increased radiosensitivity *in vitro*, clinical studies regarding the radiosensitivity in NSCLC with mutant EGFR are currently under investigation. Early results are promising and show that patients with EGFR mutation have improved locoregional control and outcomes with radiotherapy (Lee et al. 2012; Mak et al. 2011).

Beyond the EGFR itself, mutations in several EGFR downstream signaling molecules could also influence the sensitivity to EGFR-targeting agents (Fig. 6). Among them, somatic KRAS mutations are found at high rates in leukemias, colon, pancreas, and lung cancers. KRAS mutation at codon 12 or 13 has been strongly correlated with resistance to cetuximab or panitumumab therapy in a number of large studies (Lievre et al. 2006; Massarelli et al. 2007; Jimeno et al. 2009; Ready et al. 2010). In the phase III CRYSTAL study for metastatic colorectal cancers, patients with the wt KRAS treated with cetuximab plus chemotherapy showed a response rate of up to 59 % compared to 43.2 % in those treated with chemotherapy alone (Van Cutsem et al. 2009). However, there is no clear benefit to cetuximab among patients with mutated KRAS tumors with a response rate of 36.2 % compared to the 40.2 % in chemotherapy alone arm. These results led to a recommendation by ASCO in 2009 that colon cancer patients with a KRAS mutation should not receive cetuximab or panitumumab therapy (Allegra et al. 2009).

Similarly, lung cancer patients who are positive for KRAS mutation have a low response rate to gefitinib or erlotinib estimated at 5 % or less (Suda et al. 2010). Among HNSCC patients, however, the frequency of KRAS mutation is very low (Sheikh Ali et al. 2008). There is no clinical recommendation for determining the KRAS mutation status in HNSCC before applying cetuximab or panitumumab. In a recent preclinical study in UT5R9 cells, acquired resistance to cetuximab is shown to associate with the overexpression of Ras family members and the loss of radiosensitivity (Saki et al. 2013). Moreover, radioresistant UT5R9 cells were not



**Fig. 6** Mechanisms of resistance to EGFR-targeted therapy. Acquired resistance to EGFR inhibitors may develop through MET amplification and activation of EGFR/HER family members that bypass EGFR inhibition. Additional mechanisms of resistance to EGFR-targeted therapies may exist due to the selected mutations of EGFR (T790 M) and downstream effectors, such as KRAS and BRAF. Adapted with the permission from Vlacich and Coffey (2011)

radiosensitized by cetuximab, but by manipulating KARS family members. Similarly, KRAS signaling through EGFR and HRAS was shown to promote radiation survival in pancreatic and colorectal carcinoma cells (Cengel et al. 2007). Based on these results, the inhibition of KRAS signaling is suggested to be an effective approach to overcome cetuximab- and radioresistance.

## 4.2 Other Potential Mechanisms

Acquired resistance to EGFR inhibitors may result from the activation of alternative membrane-bound receptor tyrosine kinases (RTK) that bypass EGFR pathway (Fig. 6) (Camp et al. 2005). Several RTKs, such as MET (hepatocyte growth factor receptor), platelet-derived growth factor (PDGF), vesicular growth factor receptor (VEGFR), and insulin-like growth factor receptor-1 (IGF-1R), can also activate the key downstream signals of EGFR including Erk and Akt pathways that may override the inhibitory effect of EGFR inhibitors. Among them, the amplification of MET was detected in 5–20 % of lung cancers that developed acquired resistance to EGFR inhibitors (Engelman and Settleman 2008). Although MET amplification can coexist with the EGFR T790 M mutation, approximately 60 % of MET amplification is independent of T790 M mutation (Bean et al. 2007). Mechanistic studies found that cells with EGFR TKI resistance relied on MET signaling to activate Akt

through HER3-PI3 K signaling (Engelman et al. 2007; Engelman and Janne 2008). This activation of HER3-PI3 K-Akt signaling permits the cells to transmit the same downstream signaling in the presence of EGFR inhibitors. In addition, MET amplification was also found at a low frequency in patients with activating EGFR mutation prior to treatment and was associated with the subsequent development of acquired resistance to EGFR TKIs. It is very likely that EGFR inhibitor treatment may select for preexisting cells with MET amplification during the acquisition of resistance to EGFR inhibitors.

In general, EGFR T790 M and MET amplification account for ~60 % of acquired resistance to EGFR inhibitors. Other mechanisms of resistance that are operative in the remaining ~40 % of tumors are currently under active investigation. Among them, EGFR family members have been identified to play an important role in acquired resistance to EGFR inhibitors (Frolov et al. 2007; Baselga and Swain 2009; Wheeler et al. 2008) (Fig. 6). Following systematic screening of RTKs, a significant activation of HER3 was observed in cetuximab or erlotinib-resistant HNSCC and NSCLC cells in vitro (Wheeler et al. 2008). In the clinical scenario, HER3 overexpression, described in 30–80 % of primary or metastatic CRC, has also been associated with resistance to EGFR-targeting agents (Scartozzi et al. 2011). Further analysis indicates that acquired resistance to EGFR inhibitors might derive in part from activation of HER3 to effectively bypass the effect of EGFR inhibition (Jain et al. 2010). Depletion of HER3 by siRNA restores sensitivity to the EGFR inhibitors. Other than HER3, HER2 also appears to be involved in regulating acquired resistance to EGFR inhibitors (Baselga and Swain 2009; Vlacich and Coffey 2011). In HCC827 cells with acquired resistance to cetuximab, amplification of HER2 is identified as a mechanism of cetuximab resistance. Inhibition of HER2 activity or disruption of HER2/HER3 heterodimerization restores cetuximab sensitivity in both in vitro and in vivo models (Yonesaka et al. 2011). Other than these RTKs, IGF-1R and VEGFR have also been identified as crucial molecules to regulate acquired resistance to EGFR inhibitors (Viloria-Petit et al. 2001; Jones et al. 2004; Morgillo et al. 2007; Benavente et al. 2009). These findings suggest that combinations of molecular-targeted therapies blocking EGFR and other selected RTKs may offer a promising strategy to overcome acquired resistance and enhance the effectiveness of EGFR therapy.

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## 5 Next-Generation EGFR-Targeting Approaches

Acquired resistance to the first generation of EGFR-targeting agents has prompted the design and development of new-generation EGFR inhibitors. Although the underlying mechanisms for acquired resistance to EGFR inhibitors are not fully understood, several strategies to overcome the resistance have been tested based on current findings on EGFR mutation and bypass mechanism. These include the next generation of EGFR-targeting agents that target the T790 M mutation or multiple

ErbB/HER family members (Yu and Riely 2013). Agents targeting other RTK or downstream signaling have also been extensively explored.

## 5.1 More Efficient EGFR-Targeting Agents

Next-generation EGFR-targeting agents include irreversible EGFR TKIs or pan-ErbB TKIs that simultaneously target multiple members of the EGFR family. The irreversible binding mechanism may increase TKI effectiveness by prolonging the inhibition of EGFR signaling and delaying the acquisition or growth of T790 M-mutant cells. Several irreversible pan-ErbB TKIs have been developed in recent years including neratinib (HKI-272), afatinib (BIBW2992), and dacomitinib (PF00299804) (see Table 1). These agents share a 4-anilinoquinazoline structure with the ability to form covalent bonds with Cys979 residue located directly at the ATP-binding cleft of EGFR protein and thus potentially preventing T790 M mutation-related resistance (Dienstmann et al. 2012; Giaccone and Wang 2011; Dziadziuszko and Jassem 2012; Ather et al. 2013). For instance, afatinib can inhibit NSCLC tumor cell growth with either wt or mutant forms of EGFR with a 100-fold greater activity than gefitinib against L858R/T790 M double mutants (Li et al. 2008a). Preclinical studies also show that afatinib synergizes with radiation to inhibit clonogenic survival and tumor xenograft growth of bladder tumor cells (Tsai et al. 2013). A recent phase III clinical trial (LUX-Lung 3) shows that afatinib improves progression-free survival compared with cisplatin and pemetrexed as first-line treatment for lung cancer patients with EGFR mutant (Sequist et al. 2013). However, the results of other trials (LUX-Lung 1, 4) demonstrate that single agent afatinib has minimal efficacy in patients previously treated with erlotinib or gefitinib (Miller et al. 2012; Katakami et al. 2013). This lack of efficacy may be due to the high potency of afatinib against EGFR with wt or activating mutation, leading to skin rash and gastrointestinal toxicity that limited administration of necessary doses for effective EGFR T790 M inhibition (Sequist et al. 2010). Ultimately, the development of T790 M-selective EGFR inhibitors appears to be a promising strategy to reduce on-target toxicity. Recent encouraging preclinical studies identify several potent inhibitors, such as WZ4002, Gö6976, and PKC412, that display improved selectivity against EGFR T790 M over wt EGFR (Zhou et al. 2009; Lee et al. 2013). The efficiency of these inhibitors in clinical practice is as yet undefined and will be better understood from the results of several ongoing phase II/III clinical trials. Nonetheless, afatinib was recently approved in July 2013 in the USA for the first-line treatment of patients with metastatic NSCLC who have tumors with activating EGFR mutations. Afatinib has also been approved in several countries in Asia and Europe.

Development of innovative mAbs with more efficient blocking ability to EGFR is another promising strategy to overcome resistance. When two mAbs against distinct epitopes of receptor are combined, a rapid and more efficient receptor internalization is observed and followed by EGFR degradation (Friedman et al. 2005). The antibody mixture is also more effective than single antibody in

inhibiting signaling and tumor growth in tissue culture and animal models (Ben-Kasus et al. 2009; Spangler et al. 2010). Furthermore, antibody mixtures have been shown to activate complement-dependent cytotoxicity that contributes to a more effective anti-tumor capacity than single antibody (Dechant et al. 2008). Sym004 is a recently developed prototype following screening of >400 different anti-EGFR mAb combinations based on the highest anti-tumor capacity (Koefoed et al. 2011). Sym004 is a mixture of two anti-EGFR mAbs that targets non-overlapping epitopes (epitope 992 vs. 1024) in EGFR extracellular domain III. Preclinical studies demonstrate that Sym004 exhibits a superior anti-tumor capacity in comparison with cetuximab or panitumumab in both in vitro and in vivo models (Pedersen et al. 2010). Furthermore, Sym004 inhibits the growth of cancer cells with acquired resistance to cetuximab resulting from increased EGFR ligand production. Preclinical pharmacokinetic and safety studies in primates and humans indicate that Sym004 is well tolerated and does not induce unexpected toxicities (Skartved et al. 2011). A recent study shows that the powerful Sym004-induced EGFR downregulation can be translated into a profound augmentation of radiation response (Huang et al. 2013b). In HNSCC and NSCLC tumor xenografts, a superior anti-tumor capacity of Sym004 when combined with either single or fractionated radiation was observed. In addition, Sym004 demonstrated a stronger impact than cetuximab to inhibit DNA damage repair signaling and prompt the induction of apoptosis (Huang et al. 2013b). Several clinical trials are in progress to evaluate the clinical potential of Sym004 for patients with HNSCC and metastatic colorectal cancer with wt KRAS (Machiels et al. 2013; Dienstmann et al. 2013). With the known survival advantage for HNSCC patients treated with cetuximab with radiation in the phase III trial (Bonner et al. 2006), the more powerful impact of Sym004 compared to cetuximab provides a rationale to design clinical studies to test the impact of Sym004 with radiation for HNSCC patients in an effort to further improve overall outcome.

As described above, acquired resistance to EGFR inhibitors may derive in part from activation of HER2 or HER3 to effectively bypass the effect of EGFR inhibition. Dual-specific antibodies targeting both EGFR and HER3 or HER2 are currently under study. Among these, MEHD7945A is a recently developed dual-target antibody against EGFR and HER3 that shows a profound anti-tumor activity in vitro and in vivo across a variety of tumor cell types when compared to the respective monospecific antibodies (Schaefer et al. 2011). In addition, MEHD7945A is effective in facilitating antibody-dependent cell-mediated cytotoxicity, but appears to induce less skin toxicity in comparison with cetuximab in non-clinical studies (Kamath et al. 2012). Using established cetuximab- or erlotinib-resistant cells from NSCLC and HNSCC, it was found that MEHD7945A effectively regressed tumors that remain highly refractory to cetuximab or erlotinib (Huang et al. 2013a). In addition, MEHD7945A also overcomes cross-resistance to radiation in these EGFR inhibitor-resistant cells. A phase II, open-label, randomized study is currently ongoing to evaluate the efficacy and safety of MEHD7945A versus cetuximab in patients with recurrent/metastatic HNSCC who have

progressed during or following platinum-based chemotherapy. (ClinicalTrials.gov Identifier NCT01577173).

## 5.2 Target Additional Pathways

Another area of intense clinical investigation resides in the combination of EGFR-targeting agents with agents targeting other pathways involved in regulating resistance to EGFR inhibitors, such as MET and VEGFR. There are a number of agents targeting MET in clinical trials that have come to fruition recently, such as rilotumumab, cabozantinib, tivantinib, crizotinib, MetMAB, and SU11274, (Peters and Adjei 2012; Sadiq and Salgia 2013; Scagliotti et al. 2013). Among these, tivantinib and MetMAB have shown positive signals in combination with erlotinib in phase II trials of lung cancer patients and are currently being tested in phase III trials (Sequist et al. 2011a; Spigel et al. 2011). In addition, preliminary results of a phase I/II trial in chemo-refractory colorectal cancer patients also demonstrate a 10 % improvement in overall response rate with the addition of rilotumumab to panitumumab (Eng et al. 2011). Given the encouraging findings from trials of several MET inhibitors to date, blocking both EGFR and MET in combination with radiotherapy is an attractive therapeutic strategy to overcome resistance to both EGFR inhibitors and radiation. Similar to EGFR, MET is also upregulated following radiation treatment (Bhardwaj et al. 2012; De Bacco et al. 2011). Preclinical studies show that inhibition of MET using small-molecule inhibitors or siRNA can radiosensitize cancer cells. The underlying mechanism of the radiosensitizing effect of MET inhibitor may include the inhibition of MET signaling, depolarization of the mitochondrial membrane potential, impairment of DNA damage repair, abrogation of cell cycle arrest, and enhancement of apoptotic cell death (Welsh et al. 2009; Yu et al. 2012; Bhardwaj et al. 2012; Sun et al. 2013).

Another promising approach to augment EGFR inhibitor impact is to combine with agents targeting VEGFR-regulated angiogenic pathway to potentiate anti-tumor effects of EGFR inhibitors as shown in a series of preclinical studies (Bozec et al. 2009a). Several studies have also established the resultant radiosensitizing effects by the dual inhibition of angiogenesis and EGFR pathways (Bozec et al. 2007, 2008, 2009b). For example, a synergistic effect is observed in an orthotopic H&N cancer model when combining cetuximab, radiation with anti-angiogenic agents, either bevacizumab or sunitinib (Bozec et al. 2008, 2009b). In addition, use of vandetanib, a dual-targeted agent targeting both EGFR and VEGFR, enhances the therapeutic efficiency of radiation in an orthotopic lung cancer model system (Shibuya et al. 2007; Wu et al. 2007).

Although various combinations of agents show activity in preclinical studies, the application of anti-angiogenic agents with EGFR and/or radiotherapy requires careful clinical evaluation since several trials with anti-angiogenic agents have proven disappointing due to toxicity (Spigel et al. 2010; Sliesoraitis and Tawfik 2011). The combination of VEGF and EGFR inhibitors was tested in NSCLC patients using concurrent bevacizumab, erlotinib, carboplatin, paxlitaxel, and RT51.

The addition of bevacizumab/erlotinib was determined not feasible due to pulmonary hemorrhage (Stinchcombe et al. 2011). However, a phase II trial in patients with HNSCC demonstrated that the combination of bevacizumab and cetuximab was active and the overall tolerance was acceptable despite some cases of bevacizumab-related toxicities (Argiris et al. 2011). In addition, bevacizumab or vandetanib was shown to be safely incorporated in conventional chemoradiotherapy and produced encouraging efficacy for patients with locally advanced HNSCC in two recent phase I studies (Harari et al. 2011; Papadimitrakopoulou et al. 2011). Similarly, the addition of bevacizumab and erlotinib to first-line chemoradiotherapy was also feasible and produced toxicity comparable to other effective combined modality regimens for HNSCC (Hainsworth et al. 2011). These inconsistent results suggest that the application of anti-angiogenic agents to EGFR and radiotherapy in the clinic will further the clinical evaluation in a systematic treatment schema in appropriate patient groups to identify potential clinical benefits (Citrin et al. 2006).

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## 6 Conclusions

There are compelling reasons to explore the combination of radiation with agents that target growth factor receptors since radiation can activate receptor signaling. In particular, a beneficial impact of combining radiation with EGFR-targeting agents in cancer therapy has been established in recent years. This is particularly evident in HNSCC when there is phase III trial data showing a survival advantage when combining cetuximab with radiation. This clinical benefit validates preclinical data that projected a favorable interaction of anti-EGFR therapies with radiation. With over 50 % of cancer patients worldwide receiving radiation as an integral component of their treatment, this approach may provide significant stimulus to guide the combination of radiation not only with EGFR agents, but also with other molecular-targeting drugs for the improvement of cancer therapy. Many challenges remain since acquired resistance to EGFR inhibitors is emerging as a treatment barrier for optimizing EGFR-targeted therapy. With improved understanding of mechanisms of resistance, several next-generation EGFR-targeting agents have been developed and are currently being studied in preclinical and clinical models. Personalized cancer medicine based on molecular profiling of tumors is also a treatment strategy of the future. Improved selection of treatments for patients with activating or resistance mutations of EGFR or downstream effectors is central to improve the ultimate efficacy of EGFR-targeted therapies. The identification of reliable predictive markers for targeted therapies combined with radiation represents a worthy objective for the future.

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# Molecular Targeting of Integrins and Integrin-Associated Signaling Networks in Radiation Oncology

Anne Vehlow, Katja Storch, Daniela Matzke and Nils Cordes

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## Abstract

Radiation and chemotherapy are the main pillars of the current multimodal treatment concept for cancer patients. However, tumor recurrences and resistances still hamper treatment success regardless of advances in radiation beam application, particle radiotherapy, and optimized chemotherapeutics. To specifically intervene at key recurrence- and resistance-promoting molecular processes, the development of potent and specific molecular-targeted agents is demanded for an efficient, safe, and simultaneous integration into current standard of care regimens. Potential targets for such an approach are integrins conferring structural and biochemical communication between cells and their microenvironment. Integrin binding to extracellular matrix activates intracellular signaling for regulating essential cellular functions such as survival, proliferation, differentiation, adhesion, and cell motility. Tumor-associated characteristics such as invasion, metastasis, and radiochemoresistance also highly depend on integrin function. Owing to their dual functionality and their overexpression in the majority of human malignancies, integrins present ideal and accessible targets for cancer therapy. In the following chapter, the current knowledge on aspects of the tumor microenvironment, the molecular regulation of integrin-dependent radiochemoresistance and current approaches to integrin targeting are summarized.

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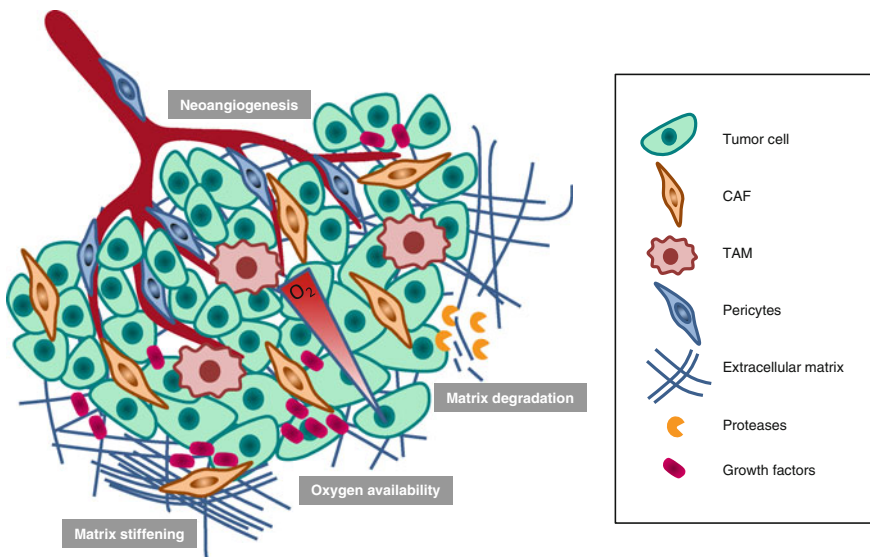
**Keywords**

Integrins · Focal adhesion signaling · Radiochemosensitization · Molecular targeting

**1 Introduction**

Radiotherapy, chemotherapy, surgery, and molecular therapeutics are essential components of current interdisciplinary treatment concepts, which are individually adapted to clinical tumor characteristics such as tumor entity, tumor volume, and metastases (Niyazi et al. 2011; Higgins et al. 2015; Searle et al. 2014; Arias 2011). In the past decades, great efforts have been made to optimize and design new irradiation techniques, which have been translated to or are on the way to clinical application (Shi et al. 2014; Allison et al. 2014). However, the success of radiation oncology will also depend on the discovery of novel molecular therapeutics that are given to patients in combination with standard radiochemotherapy. Key to a successful development of such new therapies is a deep understanding of the genetic, epigenetic, proteomic, and metabolomic tumor traits including the tumor microenvironment.

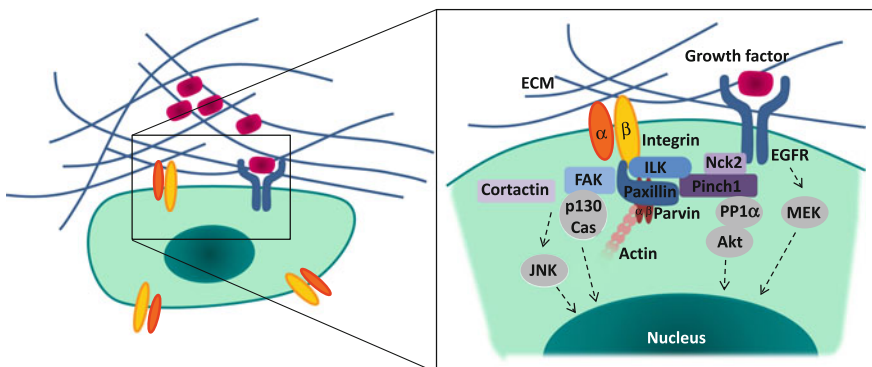
Similar to normal cells within the organism, tumor cells are constantly communicating with specialized cell types and the extracellular matrix (ECM) in their vicinity (Fig. 1). Hence, it is not surprising that the tumor microenvironment elicits tumor progression, metastatic spread, and therapy resistance (Clark and Vignjevic



**Fig. 1** Cellular and noncellular factors of the tumor microenvironment impact on therapy resistance. *CAF*, cancer-associated fibroblasts; *TAM*, tumor-associated macrophages; *O<sub>2</sub>*, oxygen

2015; Wei and Yang 2015; Barker et al. 2015; Klemm and Joyce 2014; Eke and Cordes 2015; Hanahan and Weinberg 2011). In fact, it is well known that cell adhesion to the ECM confers cell adhesion-mediated radioresistance (CAM-RR) (Cordes and Meineke 2003; Sandfort et al. 2007), and cell adhesion-mediated drug resistance (CAM-DR) (Damiano et al. 1999; Hazlehurst et al. 2000, 2006; Eke and Cordes 2011; Shishido et al. 2014; Damiano et al. 2001) for hematopoietic and solid tumors. This paradigm has recently been expanded to the whole cellular environment entitled environment-mediated drug resistance (EM-DR) (Meads et al. 2009).

Interactions of cells with ECM molecules are mainly mediated via the integrin family of heterodimeric transmembrane receptors (Hynes 2002) and regulate cell survival, cell proliferation, and cell motility (Legate et al. 2009; Harburger and Calderwood 2009). Integrins are composed of one of the 18 different  $\alpha$  and 8 different  $\beta$  chains that are non-covalently associated (Hynes 2002), and their ECM specificity is dictated by the 24 possible  $\alpha/\beta$  pairings (Humphries et al. 2006). Together with growth factor receptors, such as the epidermal growth factor receptor (EGFR), integrins translate extracellular cues to intracellular signals in specialized cell membrane areas, so-called focal adhesions (FA) (Geiger and Yamada 2011; Storch and Cordes 2012; Geiger et al. 2009). By this mechanism, integrins not only function to provide mechanical anchorage and chemical signals to cells (outside-in activation), but also to respond to intracellular stimuli by changing their conformation and ECM ligand affinity (inside-out activation) (Calderwood 2004). These two functionalities are mediated by a multiplicity of intracellular adaptor and signaling proteins that are collectively termed the “adhesome” (Zaidel-Bar et al. 2007; Geiger and Zaidel-Bar 2012). Importantly, adaptor proteins such as talin and kindlin directly bind to the cytoplasmic tails of integrins and recruit further signaling mediators to the FA (Morse et al. 2014). Among these, the focal adhesion kinase (FAK) and the IPP complex consisting integrin-linked kinase (ILK), particularly



**Fig. 2** Molecular insight in integrin and epidermal growth factor receptor (EGFR) signaling pathways involved in radiochemoresistance. *FAK*, focal adhesion kinase; *PINCH1*, particularly interesting new cysteine-histidine-rich protein 1; *ILK*, integrin-linked kinase; *AKT*, alpha serine/threonine-protein kinase; *p130Cas*, p130 Crk-associated substrate; *MEK*, mitogen-activated protein kinase kinase; *JNK*, c-Jun N-terminal kinase; *PP1α*, protein phosphatase 1α; *Nck2*, non-catalytic region of tyrosine kinase 2

interesting new cysteine-histidine-rich protein 1 (PINCH1) and  $\alpha$ -parvin, bridge the gap to intracellular signaling cascades and recruit and modulate actin filaments through Rho GTPases (Legate et al. 2006; Iwamoto and Calderwood 2015) (Fig. 2).

The significance of the ECM-integrin connection as determinant of tumor cell radio- and chemosensitivity and the potential of integrins as biomarkers and prognostic factors have made them potential cancer targets (Cordes and Park 2007; Hehlhans et al. 2007; Goodman and Picard 2012; Vehlow and Cordes 2013). Understanding the molecular mechanisms underlying FA hub signaling with regard to therapy sensitivity will foster the development of feasible multimodal treatment strategies. In the following chapters, current knowledge of the tumor microenvironment and the impact of integrin signaling on treatment resistance are reviewed.

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## 2 The Microenvironment and the Modulation of Radiochemoresistance

During the past decade, a multitude of big-scale high-throughput screens has been performed to identify new drugs for combination with conventional radiochemotherapy to cure cancer patients (Collins and Workman 2006). However, potent anticancer drugs frequently fail in clinical trials because these preclinical screens are mainly performed without consideration of the tumor microenvironment (TME) (Jain 2013). Recent evidence suggests that the TME determines the success of radiochemotherapy by promoting therapy resistances (Barker et al. 2015; Barcellos-Hoff and Cordes 2009; Tavora et al. 2014; Nakasone et al. 2012). The underlying molecular mechanisms remain unclear.

Certainly, a tumor is more than an accumulation of malignant cells, it resembles a complex tissue consisting of different cell types such as stromal (e.g., cancer-associated fibroblasts (CAF) and pericytes), endothelial, and immune cells (e.g., tumor-associated macrophages and lymphocytes) (Hanahan and Weinberg 2011; Gandellini et al. 2015; Egeblad et al. 2010). In addition to these cellular components, the TME is furthermore defined by several noncellular factors such as the availability of oxygen, chemokines, growth factors, metabolites, and the surrounding ECM, which altogether create a unique niche for cancer cells (Hanahan and Weinberg 2011; Jain 2013) (Fig. 1). In normal tissues, the stroma provides connective and structural support and maintains physiological homeostasis in a tumor suppressive manner. Once transformation has commenced, stromal cells can facilitate cancer progression (Rønnov-Jessen et al. 1996; Junttila and de Sauvage 2013; Chen et al. 2015). During the neoplastic process, the TME is infiltrated by CAF and myofibroblasts (Gandellini et al. 2015; Chen et al. 2015; Micke and Ostman 2005; Kalluri and Zeisberg 2006; Sanchez-Lopez et al. 2015) leading to ECM stiffening and remodeling by the secretion of ECM proteins such as collagens and matrix metalloproteinase (MMP) enzymes (Egeblad et al. 2010; Kessenbrock et al. 2010; Levental et al. 2009). Thereby, the biochemical ECM properties are dramatically altered resulting in a biophysical, stiffness-related plasticity fundamentally different from the corresponding normal tissue (Levental et al. 2009;

Frantz et al. 2010; Butcher et al. 2009). These perturbations are known to facilitate tumorigenesis and metastasis by altering the mechanotransductive equilibrium between cells and their surrounding matrix (Plodinec et al. 2012; Kumar and Weaver 2009). Therefore, novel targeting strategies should not only reflect the complexity of different cell types within the TME but also their interaction with TME components to gain profound clinical benefits (Paulsson and Micke 2014). Further efforts are necessary to identify new potential targets that help to normalize the TME and simultaneously sensitize tumor cells.

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### **3 The Modulation of Radiochemoresistance by Integrins and Focal Adhesion Signaling Networks**

In contrast to normal tissues, the expression of many integrins is altered in tumor cells of different entities as well as tumor-associated endothelial cells or CAFs, leading to changes in cellular behavior and an aggressive tumor phenotype (Barker et al. 2015; Danen 2005; Guo and Giancotti 2004; Desgrosellier and Cheresch 2010). Facilitating tumor cell adhesion and invasion as obligate part of most of the integrin heterodimers, altered  $\beta 1$  integrin expression is detected in breast carcinoma (Zutter et al. 1993), colon carcinoma (Koretz et al. 1991; Stallmach et al. 1992; Fujita et al. 1995), pancreatic carcinoma (Shimoyama et al. 1995; Hall et al. 1991), head and neck squamous cell carcinoma (HNSCC) (Jones et al. 1993; Eriksen et al. 2004), and glioblastoma multiforme (GBM) (Paulus et al. 1993). Interestingly, integrin receptors containing the  $\beta 4$ ,  $\beta 5$ ,  $\beta 6$ , and  $\beta 8$  subunits are often associated with a metastatic potential (Stallmach et al. 1992; Hall et al. 1991; Eriksen et al. 2004; Koukoulis et al. 1991; Vogetseder et al. 2013; Gui et al. 1996; Previtali et al. 1996), while overexpressed  $\alpha v\beta 3$  and  $\alpha v\beta 5$  critically function in angiogenesis and metastasis to distant organs (Koukoulis et al. 1991; Vogetseder et al. 2013; Gui et al. 1996; Weis and Cheresch 2011; Gingras et al. 1995).

Apart from tumor-associated integrin alterations, irradiation has been shown to increase integrin expression levels in a dose-dependent manner in several cancer entities such as GBM (Cordes and Meineke 2003; Wild-bode et al. 2001; Wick et al. 2002; Rieken et al. 2011; Eke et al. 2012a; Cordes et al. 2003), breast cancer (Park et al. 2003, 2006), NSCLC (Cordes et al. 2002), colon carcinoma (Meineke et al. 2002) and pancreatic carcinoma (Cordes and Meineke 2003). This phenomenon facilitates tumor cell survival and anti-invasion after X-ray exposure and corresponds to increased radiochemoresistance, pinpointing a critical function of integrins in the tumor cell response to irradiation (Damiano et al. 1999; Hazlehurst et al. 2000; Shishido et al. 2014; Damiano et al. 2001; Hazlehurst et al. 2006; Cordes and Meineke 2003). How exactly integrin expression is regulated upon irradiation is not well understood. Different independent studies suggest a positive feedback mechanism between nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- $\kappa$ B) and integrin expression that can be targeted to enhance the tumor cell therapy response (Ritchie et al. 2000; Bradbury et al. 2001; Ahmed et al. 2013). Taken together, current data support the concept of therapeutic integrin targeting as

tumor-associated and radiation-dependent changes in integrin expression are critically linked to different stages of tumor progression, radiochemoresistances, and the clinical outcome (Cordes and Park 2007; Goodman and Picard 2012; Eke and Cordes 2015; Hehlhans et al. 2007).

### 3.1 Integrin-Dependent Radiochemoresistance

Among the 24 possible integrin heterodimers, especially those receptors containing the  $\beta 1$  subunit and associated signaling hubs are the key components regulating resistance to therapy (Eke and Cordes 2015; Cordes and Park 2007; Janes and Watt 2006). In HNSCC, several recent studies demonstrate that  $\beta 1$  integrin targeting with inhibitory antibodies or siRNA results in radiosensitization of tumor cells *in vitro* and *in vivo* (Eke and Cordes 2015; Dickreuter et al. 2015; Eke et al. 2012b, c). Especially in three-dimensionally (3D) grown tumor HNSCC cells,  $\beta 1$  integrin targeting reduces proliferation and the repair of radiogenic DNA double-strand breaks (Dickreuter et al. 2015; Eke et al. 2012c) by deactivation of FAK signaling as early key mechanistic event (Dickreuter et al. 2015). Also cells from GBM respond to an inhibition of  $\beta 1$  integrin with reduced clonogenic survival upon irradiation (Eke et al. 2012a; Cordes and Meineke 2003) by a hampered DNA double-strand break repair (Eke et al. 2012a). Other preclinical studies show that  $\beta 1$  integrin is one of the mediators of glioblastoma cell invasion (Vehlow and Cordes 2013; Cordes and Meineke 2003; Carbonell et al. 2013) and exerts its function via the regulation of different pro-invasive signaling modules such as MMP-2 activity and FAK (Cordes and Meineke 2003; Carbonell et al. 2013). Similar results are shown for breast carcinoma cells. Here,  $\beta 1$  integrin inhibition decreases 3D tumor cell proliferation and induces apoptosis by downregulating the Akt signaling pathway without affecting nonmalignant cells (Park et al. 2006, 2008). In addition,  $\beta 1$  integrin inhibition allows a reduction of the irradiation dose to achieve comparable tumor growth inhibition and apoptosis in breast carcinoma xenografts (Park et al. 2008), suggesting a possible therapeutic benefit.

Apart from  $\beta 1$  integrin, much attention has been focussed on  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrin heterodimers due to their prominent role in angiogenesis and neovascularization of tumors (Brooks et al. 1994; Eliceiri and Cheresh 2000). In GBM, these integrins are linked to tumor progression correlating with the histological tumor grade (Gingras et al. 1995; Gladson and Cheresh 1991; Bello et al. 2001; Schnell et al. 2008) and a poor overall survival (Ducassou et al. 2013). Importantly, it has been shown that the inhibition of both heterodimers induces radiosensitization of glioblastoma cells *in vitro* by the induction of mitotic catastrophe (Monferran et al. 2008). This radiosensitizing effect further involves ILK signaling leading to the activation of the small GTPase RhoB (Monferran et al. 2008). Also additional preclinical studies suggest a positive effect of  $\alpha v\beta 3$  and  $\alpha v\beta 5$  therapeutic antagonists. Application of the  $\alpha v\beta 3$  peptide antagonist S247 enhances anti-angiogenic and anti-tumor effects in combination with fractionated radiotherapy (Abdollahi et al. 2005). Cilengitide, an  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrin antagonizing arginine-glycine-aspartic acid (RGD) peptide, modulates adhesion and viability of glioblastoma cells

in vitro and increases animal survival in a schedule-dependent manner in combination with irradiation (Maurer et al. 2009; Mikkelsen et al. 2009). The data suggest that this effect of cilengitide is partially determined by an induction of apoptosis of endothelial and glioblastoma cells and can be further enhanced when combined with temozolomide (TMZ) therapy (Oliveira-Ferrer et al. 2008).

In addition to a plethora of studies investigating  $\beta$  integrins as potential anti-cancer targets, only little attention has been focused on the  $\alpha$  integrin subunits (Kurokawa et al. 2008; Nam et al. 2010; Nagata et al. 2013; Shirakihara et al. 2013; Zhou et al. 2014; Steglich et al. 2015). Similar to  $\beta$  subunits,  $\alpha$  subunits are also highly upregulated in HNSCC, GBM, and other entities (Vehlow and Cordes 2013; Estilo et al. 2009; Peng et al. 2011). Intriguingly, from the four  $\alpha$  integrin subunits ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ , and  $\alpha 6$ ) coprecipitated with inhibited  $\beta 1$  integrin, only targeting of  $\alpha 3$  integrin resulted in enhanced radiosensitivity of HNSCC cell lines (Steglich et al. 2015). A partial independence of  $\alpha 3$  integrin from  $\beta 1$  integrin signaling was obvious by radiosensitization when  $\beta 1$  and  $\alpha 3$  integrins were co-targeted (Steglich et al. 2015). In the future, more research is warranted to clarify how therapy resistance mechanisms are induced by different  $\alpha$  and  $\beta$  integrins and whether these can be circumvented by therapeutic interventions.

### 3.2 Adhesome Components and Radiochemoresistance

As integrin receptors are devoid of own enzymatic activity, the association with adaptor proteins in FA is critical to translate information from the extracellular microenvironment into intracellular signaling cues (Legate et al. 2009) (Fig. 2). By activating specific pro-survival signaling mechanisms, integrins essentially contribute to the evasion of apoptotic cell death and the acquisition of resistance to radiation and chemotherapy.

As main mediator of integrin signaling, several studies have implicated FAK as the most prominent regulator of tumor progression and radioresistance (Eke et al. 2012b; Tilghman and Parsons 2008). In fact, overexpression of FAK has been linked to poor prognosis in several tumor entities (Miyazaki et al. 2003; Fujii et al. 2004; Lark et al. 2005). FAK is recruited to FA upon binding of integrins to the ECM, and its kinase activity is activated by autophosphorylation on tyrosine 397 (Schaller 1992, Parsons 2003). Interaction with various signaling proteins such as p130 Crk-associated substrate (p130Cas) facilitates further intracellular signal transduction events (Polte and Hanks 1995). Targeting of FAK for cancer therapy is a promising approach as siRNA or small molecule inhibitors induce apoptosis and tumor growth delay of HNSCC (Eke and Cordes 2011; Hehlhans et al. 2009), GBM (Golubovskaya et al. 2013; Roberts et al. 2008), NSCLC (Roberts et al. 2008), and pancreatic carcinoma (Hochwald et al. 2009). Moreover, FAK inhibition elicits radiosensitization (Eke and Cordes 2011; Eke et al. 2012b; Hehlhans et al. 2009; Hehlhans et al. 2012). Effector molecules of FAK that regulate its radiosensitizing potential include the Akt/mitogen-activated protein kinase kinase 1/2 (MEK1/2) (Hehlhans et al. 2012), Cortactin/c-Jun N-terminal kinase (JNK) (Eke et al. 2012b),

and PINCH1/protein phosphatase 1 $\alpha$  (PP1 $\alpha$ ) signaling axes (Eke et al. 2010). Also the inactivation of FAK in endothelial cells seems a promising approach as this strategy increased chemosensitivity by suppressing NF- $\kappa$ B-dependent cytokine secretion (Tavora et al. 2014).

In addition to FAK, integrins link to a ternary protein complex consisting of ILK, PINCH1, and  $\alpha$ -parvin (Legate et al. 2006), which transmits integrin outside-in signals (Legate et al. 2006). Recent studies suggest that ILK mainly serves as adaptor protein as evidence for enzymatic function is lacking in vivo (Wickström et al. 2010; Qin and Wu 2012). Regarding cellular radiosensitivity, ILK clearly plays a pro-survival role in tumor cells and is dispensable for radiation survival of fibroblasts (Hehlhans et al. 2008). In addition, overexpression of wildtype ILK or ILK mutants results in radioresistance of leukemia and NSCLC.

In contrast to ILK, knockdown of PINCH1 using siRNA technology generally radiosensitizes tumor cells from several cancer entities including colorectal, lung, pancreatic, and cervix cancer (Eke et al. 2010; Sandfort et al. 2010). Great part of the underlying mechanism has been identified indicating direct PINCH1 interactions with PP1 $\alpha$  as key determinant of Akt pro-survival signaling. Thus, PINCH1 is considered a promising cancer target (Eke et al. 2010).

Another function of the IPP complex is the functional linkage of integrin-associated signaling pathways to receptor tyrosine kinases, such as the EGFR (Legate et al. 2006). Both signaling hubs cooperatively regulate survival, proliferation, adhesion, and migration of cells (Yamada and Even-Ram 2002) and significantly contribute to HNSCC radiochemotherapy resistance (Eke and Cordes 2015; Eke et al. 2013, 2015; Rossow et al. 2015). The exact interaction of both classes of cell surface receptors is still unclear, but evidence suggest that certain adaptor proteins, such as non-catalytic region of tyrosine kinase 2 (Nck2) connect integrins and EGFR via the IPP complex on the molecular level (Legate et al. 2006) and stimulate the repair of therapy-induced DNA double-strand breaks (Rossow et al. 2015). In addition, recent data from 3D IrECM grown HNSCC cells show that integrin signaling attenuates the effects of an EGFR targeted therapy with Cetuximab through bypassing JNK interacting protein 4 (JIP4) and JNK2 signaling (Eke et al. 2013). These data clearly indicate crosstalk of both receptor classes. Furthermore, preclinical research apparently demonstrates that co-targeting of  $\beta$ 1 integrin and EGFR in combination with radiotherapy is a potent strategy to overcome resistance in HNSCC by modulating a protein complex consisting of FAK and extracellular signal-regulated kinase (Erk) and associated signaling hubs (Eke and Cordes 2015).

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#### **4 Clinical Trials Involving Molecular-Targeted Drugs for Integrins and Adhesome Components**

Owing to their increased expression on tumor cells and fundamental functions in tumor-associated normal cells, integrins are considered encouraging cancer targets. Another tremendous advantage of integrins is their cell surface localization, making them accessible targets for function-blocking monoclonal antibodies and



antagonistic peptides for abolishing pro-survival signaling mechanisms upon binding to the integrin extracellular domain. Whereas integrin targeting is already successfully implemented in the therapy of some human diseases such as multiple sclerosis (natalizumab,  $\alpha 4$  integrin) and ischemia (abciximab,  $\beta 3$  integrin) (Salzler et al. 2015; Gensicke et al. 2012; Horsley et al. 2015; Coles 2015), potential anti-integrin components are still tested in clinical trials to clarify the overall effectiveness as anti-cancer therapeutics. In total, 65 inhibitory anti-integrin compounds have been generated for therapy of human diseases; however, only five entered late phase clinical trials on cancer (Table 1). Noteworthy, all of these inhibitors target RGD-binding integrins either by blocking specific integrin receptors or integrin heterodimers containing the  $\alpha v$  subunit (Goodman and Picard 2012).

Etaracizumab (MEDI-522) is one of the first humanized  $\alpha v \beta 3$  antagonists, and its precursor vitaxin has been tested in phase I and phase II clinical trials (Gutheil 2000; Delbaldo et al. 2008; Hersey et al. 2010; McNeel 2005). Besides anti-angiogenic properties and low toxicity (Gutheil 2000), some patients with advanced solid tumors, i.e., renal cell carcinoma (RCC) and melanoma, benefited from a disease stabilization after vitaxin treatment (Delbaldo et al. 2008; Hersey et al. 2010; McNeel 2005). However, inhibition of  $\alpha v$ -containing integrins with the monoclonal antibody intetumumab did not improve progression-free survival of stage IV melanoma patients alone or in combination with the standard treatment using dacarbazine (O'Day et al. 2011). Nevertheless, a slight but not significant enhancement of overall survival of stage IV melanoma patients was apparent after intetumumab administration requesting for further clarification in larger cohorts (O'Day et al. 2011). Cilengitide is the first integrin antagonist tested in a phase III clinical trial. After early disappointing results in pancreatic cancer, melanoma, and HNSCC, a promising antitumor activity in newly diagnosed and recurrent GBM patients was shown in phase I and phase II clinical studies applying cilengitide (Reardon et al. 2008). Finally, the subsequent phase III CENTRIC study shows no improvement of patient survival by cilengitide on top of standard radiochemotherapy (Stupp et al. 2014) questioning the general clinical impact of  $\alpha v$  integrin antagonists.

**Table 1** Integrin inhibitors in phase II and phase III clinical trials for cancer therapy

Clinical phase	Target	Drug	Tumor entity	References
Phase II	$\alpha 5 \beta 1$	ATN-161	HNSCC, GBM	Barkan and Chambers (2011)
		Volociximab	Melanoma, RCC, ovarian cancer, peritoneal neoplasms	Goodman and Picard (2012); Bell-McGuinn et al. (2011)
	$\alpha v \beta x$	Intetumumab	Melanoma	O'Day et al. (2011)
	$\alpha v \beta 3$	Etaracizumab	Melanoma, RCC, other solid tumors	Hersey et al. (2010), McNeel (2005)
Phase III	$\alpha v \beta 3$ , $\alpha v \beta 5$	Cilengitide	GBM	(Reardon et al. (2008), Stupp et al. (2014)



Volociximab, a function-blocking antibody and inhibitor of the  $\alpha 5 \beta 1$  fibronectin receptor, had no toxicity in a clinical phase I study involving patients with melanoma and renal cell carcinomas (Ricart et al. 2008; Heng et al. 2010). Volociximab failed in combination with chemotherapy in a later phase II trial on ovarian and primary peritoneal cancer patients, and the study was terminated due to a lack of efficacy (Goodman and Picard 2012; Bell-McGuinn et al. 2011). Another inhibitor of  $\alpha 5 \beta 1$  integrin is the non-RGD-based peptide ATN-161, which showed no risks in phase I clinical trials (Cianfrocca et al. 2006). To clarify whether ATN-161 has any effects in combination with radio- and chemotherapy, further phase II trials in HNSCC and GBM patients are planned (Thundimadathil 2012; Barkan and Chambers 2011).

Despite our knowledge of integrin function in tumor development, progression, resistance, and metastasis, targeting integrins was not a clear success story until now. One might ask why and take into consideration a number of causes. For example, integrins might be influenced by other transmembrane receptors, integrin expression might be more cytoplasmic than membranous, receptor tyrosine kinase and cytoplasmic protein kinase bypass signaling might be fostered by the inhibition of specific integrins, ineffective inhibitor binding to integrins, and so on. An alternative to block integrins is the deactivation of integrin-associated signaling mediators using small molecules. As the key molecule transducing integrin signals, FAK can be targeted by either inhibiting its activity, its autophosphorylation, or scaffold function. Whereas a couple of preclinical trials with inhibitors for FAK enzymatic activity showed a decreased tumor cell growth in pancreatic (Liu et al. 2008) and head and neck cancers (Hehlgans et al. 2009), first FAK inhibitors are currently tested in phase I clinical trials.<sup>1</sup> Clinical application of the FAK inhibitor PF-562,271, for example, resulted in stable disease in patients with pancreatic cancer supporting the notion of FAK as auspicious therapeutic target (Infante et al. 2012). Many more FAK kinase inhibitors are currently in clinical trials for which results are long awaited.

Taken together, despite intensive development during the last decades, there is currently no therapeutic available that effectively targets integrins or adhesion components with profound clinical benefit. It seems clear that integrin inhibitors will not be curative by themselves provoking them as adjuvants to conventional cancer regimens.

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## 5 Future Perspectives

With the increasing desire for individualized therapy regimens, the need for the identification of new biomarkers becomes apparent. In this regard, integrins and adhesion components have been demonstrated to substantially contribute to tumor development, progression, and resistance as well as correlated to reduced therapy sensitivity of certain tumor cell subpopulations and clinical outcome. Therefore,

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<sup>1</sup>ClinicalTrials.gov. <https://clinicaltrials.gov/>.

novel targeting strategies may take into consideration and reflect the high complexity of cellular and noncellular components faced in a solid cancer. Currently, coherent data on integrins and adhesion expression, functionality and involvement in progression of different tumor entities are lacking and need to be determined using a combination of high sophisticated approaches such as phosphoproteomics and proteomics, metabolomics, epigenetics, and system biology. For the development of novel integrin-based therapeutic strategies, clinicopathological and treatment data require our attention to obtain a comprehensive understanding of integrin and adhesion regulation during tumor progression and therapy.

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# Personalized Radiation Oncology: Epidermal Growth Factor Receptor and Other Receptor Tyrosine Kinase Inhibitors

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## Abstract

Molecular biomarkers are currently evaluated in preclinical and clinical studies in order to establish predictors for treatment decisions in radiation oncology. The receptor tyrosine kinases (RTK) are described in the following text. Among them, the most data are available for the epidermal growth factor receptor (EGFR) that plays a major role for prognosis of patients after radiotherapy, but seems also to be involved in mechanisms of radioresistance, specifically in repopulation of tumour cells between radiotherapy fractions. Monoclonal

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antibodies against the EGFR improve locoregional tumour control and survival when applied during radiotherapy, however, the effects are heterogeneous and biomarkers for patient selection are warranted. Also other RTK's such as c-Met and IGF-1R seem to play important roles in tumour radioresistance. Beside the potential to select patients for molecular targeting approaches combined with radiotherapy, studies are also needed to evaluate radiotherapy adaptation approaches for selected patients, i.e. adaptation of radiation dose, or, more sophisticated, of target volumes.

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**Keywords**

EGFR, Biomarker, Radiotherapy, HER-2, Receptor tyrosine kinases

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## 1 Introduction

Treatment decisions in clinical radiation oncology are today based on general predictive biomarkers such as tumour histology or tumour mass (macroscopic tumour vs. postoperative residuals). Beside indications for radio- or radiochemotherapy or total radiation dose, also decisions on fractionation schedules can be based on such factors. Examples are squamous cell carcinoma of the head and neck (HNSCC) that express a considerable time factor, i.e. improvement of local tumour control at shorter overall treatment time by repopulating cancer stem cells during treatment, and are thus better treated using accelerated radiation treatment schedules. Recent data showed that also tumour size can impact the outcome after different fractionation schedules with a larger time factor for larger compared to smaller non-small-cell lung cancer (Soliman et al. 2013). Beyond these "classical" biomarkers, there are currently no molecular biomarkers in clinical use as a basis for treatment decisions in radiation oncology. However, a number of promising candidate biomarkers are currently being tested in preclinical and clinical studies. Notably, such biomarkers need to be evaluated separately for radio-oncological combined treatment approaches as compared to drug treatments alone. The reasons are the different endpoints (mostly curative for combined treatment, palliative for systemic treatment alone in solid tumours) and the potential interactions between both treatment modalities when combination schedules are used.

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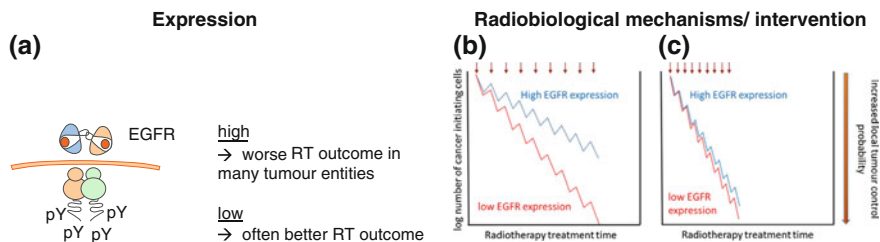
## 2 Epidermal Growth Factor Receptor and Other Receptor Tyrosine Kinases

### 2.1 Importance of EGFR for Radiotherapy Outcome

The ErbB receptor family presents one of about 20 known subfamilies of the receptor tyrosine kinase (RTK) receptors. It includes four receptor subtypes (EGFR/HER1/ErbB1, HER2/neu/ErbB2, HER3/ErbB3 and HER4/ErbB4), of which the epidermal

growth factor receptor (EGFR) is the best-evaluated receptor. Compared to the normal tissues of the tumour's origin, the EGFR is the most frequently overexpressed receptor in human tumours. A prominent example is squamous cell carcinoma of the skin and head and neck, where 80–100 % of the tumours were found to show a high EGFR expression, but also carcinoma of the cervix uteri (80 %), endometrial cancer (90 %), non-small-cell lung cancer (40–80 %) and glioblastoma (40–50 %) belong to the high EGFR-expressing tumours [reviewed in Salomon et al. (1995)]. A high EGFR expression has been reported to be associated with lower tumour control rates after radiotherapy for several tumour entities (Nicholson et al. 2001; Baumann et al. 2007); however, there are also contradictory results showing no or only weak or even reverse associations between both parameters (Chakravarti et al. 2005a; Chakravarti et al. 2005b; Lee et al. 2005; Marioni et al. 2011). It needs to be mentioned that an association of the EGFR expression with survival or tumour control has also been shown for other oncologic treatment modalities, supporting a prognostic rather than predictive value of the protein expression: in addition to retrospective data on patient outcome after surgery or chemotherapy of different tumour entities, a recent analysis of head and neck squamous cell cancer (HNSCC) patients treated within two different prospective clinical trials either with surgery without an EGFR-targeted agent or with radiochemotherapy in combination with the anti-EGFR antibody cetuximab has shown that a high EGFR protein expression and elevated phosphorylation of the tyrosine kinase at Y1068 correlated with lower survival, supporting a prognostic value independent on the kind of treatment (Wheeler et al. 2012). Interestingly, evaluation of HNSCC patients enrolled in a randomized RTOG trial revealed an independent prognostic value of EGFR protein expression for overall survival, disease-free survival and local tumour control, but not for metastases-free survival (Ang et al. 2002), suggesting that the reason for the different response is indeed local tumour resistance and not metastatic potential. These data support strategies on local treatment intensification to overcome this resistance.

The EGFR seems to be involved also in mechanisms of radiation resistance, specifically in repopulation of cancer stem cells between radiation fractions. Repopulation determines an increase of the number of cancer stem cells between irradiation fractions by accelerated proliferation and/or by decreased cell loss. It causes the so-called time factor of radiotherapy that is the decrease of tumour cure probability with increasing overall treatment time. Although heterogeneity exists between different tumour models, a coincidence between onset of repopulation and increase of membranous EGFR protein expression has been shown experimentally in FaDu xenografts (Eicheler et al. 2005; Petersen et al. 2003). The causal relationship of EGFR expression and repopulation is supported by post hoc analyses of clinical randomized trials. Both in the CHARTWEL-bronchus as well as in DAHANCA trials, non-small-cell lung cancer or HNSCC has been treated in different overall treatment times. Both post hoc analyses showed that specifically tumours with high EGFR expression have significantly lower local tumour control probabilities when overall treatment times are prolonged and that this disadvantage can be partly or completely dissolved with shortening overall treatment time (Bentzen et al. 2005; Eriksen et al. 2005). This mechanism is schematically shown



**Fig. 1** EGFR protein expression is often associated with radiotherapy outcome, i.e. locoregional tumour control or survival **a** The EGFR seems to be involved in biological mechanisms of radiation resistance, specifically repopulation of cancer stem cells **b, c**. After each radiation fraction ( $\downarrow$ ), a percentage of the cancer (stem) cells are inactivated, but the surviving cancer stem cells repopulate within the time interval to the next fraction, thus again increasing their number. This repopulation is obviously more distinct in high EGFR-expressing tumours, leading to lower tumour control probabilities in this subgroup **(b)**. Applying the radiotherapy treatment schedule within a shorter overall treatment time **(c)** reduces the amount of repopulation and outweighs this disadvantage for high EGFR-expressing tumours

in Fig. 1. Thus, promising experimental interventions include not only local treatment modifications by radiation dose, but also modifications of the radiotherapy fractionation schedule, i.e. the application of accelerated radiotherapy specifically in high EGFR-expressing tumours.

EGFR expression has been shown to be associated also with expression of other RTKs, and consideration of both may help to refine different prognostic groups of patients. Retrospective data on non-small-cell lung cancer suggest that a genetic amplification of the EGFR measured by fluorescence in situ hybridization (FISH) is more frequent in squamous cell carcinoma and is associated with an amplification of the insulin-like growth factor 1 receptor (IGF-1R, see below), and the amplification of both receptors or a protein overexpression defines a patient group with lower disease-free survival (DFS) (Ludovini et al. 2013; Gately et al. 2014).

## 2.2 Importance of Other ErbB Receptors for Radiotherapy Outcome

ErbB2 (HER2) is frequently overexpressed in breast cancer but also in a variety of other cancers. In clinical data sets, ErbB2 seems to be associated with higher tumour stage and aggressiveness in different tumour entities (Salomon et al. 1995); however, it is not clear whether this phenomenon has any association to resistance to specific treatments or is just a feature of these tumours. Overexpression of ErbB2 together with the putative cancer stem cell marker CD44 has been shown to be more frequent in breast cancer recurrences compared to primary breast cancer (Duru et al. 2012). In vitro, HER2+/CD44+/CD24-/low MCF7/C6 cells showed elevated aldehyde dehydrogenase (ALDH) activity, invasion and sphere formation as well as faster tumour formation after cell inoculation in vivo. After irradiation in vitro, clonogenic

cell survival was lower in HER2 siRNA knock-down cells compared to HER2 positive cells, indicating a higher radioresistance in HER2 positive cells (Duru et al. 2012). These data suggest a potential value of ErbB2 as a marker of tumour aggressiveness and radioresistance that needs to be validated in clinical data sets.

For ErbB3, there are some clinical data suggesting a prognostic role of a high expression with clinical outcome in colorectal cancer (Ledel et al. 2014). In ErbB2 positive breast cancer, low ErbB3 expression has been shown to be associated with better progression-free survival compared to high ErbB3-expressing tumours (Lipton et al. 2013). Also here, there is currently no evidence for specific interaction with cancer treatments. However, preclinical data suggest that compensatory ErbB3 can be activated when EGFR signalling is blocked and that this activation, via src signalling, increases radioresistance by preventing apoptosis in breast cancer cells *in vitro* (Contessa et al. 2006).

Little is known about the importance of ErbB4 for cancer prognosis and specifically for radiation resistance. While few mostly preclinical data sets suggest an importance of specific mutations for tumour aggressiveness, there are no valid data on a potential value of the receptor expression or mutations for treatment resistance.

### **2.3 Importance of EGFR and Its Downstream Signal Transduction for the Outcome of Combined Radiotherapy and EGFR-Targeted Agents**

For EGFR targeting, specifically for the application of EGFR tyrosine kinase inhibitors outside the context of radiotherapy, some biomarkers have been established that can predict tumour response to treatment [reviewed in Krause and Baumann (2008)]. One example are specific mutations of the EGFR tyrosine kinase that are mostly in-frame deletions in exon 19 (codon 746–750), missense mutations in exon 21 (codon 858) or missense or insertion mutations in exon 18–21 [reviewed in Johnson and Janne (2005)]. EGFR gene amplification measured by FISH can be shown in about 30 % of non-small-cell lung cancer (NSCLC). While NSCC with EGFR amplification seems to have a generally worse prognosis (Hirsch et al. 2003; Jeon et al. 2006), this can be counteracted by the application of EGFR-TK inhibitors (Hirsch et al. 2006; Tsao et al. 2005; Temam et al. 2007), supporting a predictive value of high EGFR gene copy numbers for EGFR-TK inhibition alone. The EGFR downstream signal transduction molecule RAS plays a role in mediating radio- and chemotherapy resistance (Bernhard et al. 2000; Chakravarti et al. 2002). In contrast to KRAS wildtype tumours, antiproliferative effects of EGFR-TK inhibitors are not evident in tumours harbouring activating KRAS mutations. Beyond several retrospective data sets, this is supported by results from clinical randomized phase III trial on patients with NSCLC and colorectal cancer, where a correlation of activating KRAS mutations with shorter survival time and time to progression after EGFR-TK inhibition or application of anti-EGFR antibodies has been established (Eberhard et al. 2005; Bokemeyer et al. 2009; Karapetis et al. 2008).

In the context of combined EGFR inhibition with radiotherapy, so far no specific molecular biomarker has been established for clinical use. This is in parts caused by the fact that the group of EGFR-TK inhibitors have not shown any improvement in the curative effect of radiotherapy when both treatments are applied simultaneously or sequentially. Thus, biomarkers are currently only needed for combined radiotherapy and anti-EGFR antibodies, i.e. for simultaneous radiotherapy and cetuximab as the currently only approved treatment combination. Here, the combined treatment improves local tumour control and survival in patients with HNSCC when compared to radiotherapy alone (Bonner et al. 2006; Bonner et al. 2010). However, the average effect seems to be not superior to standard radiochemotherapy (Caudell et al. 2008). In the light of sometimes impressive responses of individual tumours to the combination of radiotherapy and cetuximab, predictive markers that help to stratify patients for the treatment with radiotherapy plus cetuximab versus radiochemotherapy are urgently needed, because stratification alone would be expected to increase local tumour control rates and survival in this situation. Post hoc analyses of the above-mentioned trial are ongoing with so far no promising biomarkers reported. Preclinical analyses showed a correlation of genetic EGFR amplification measured by FISH with the improvement of local tumour control by cetuximab applied simultaneously to irradiation, whereas tumours without amplification show very heterogeneous response (Gurtner et al. 2011). Also tumour micromilieu parameters like perfusion appear to be potential candidate biomarkers (Gurtner et al. 2013). Using a theragnostic approach in preclinical experiments, i.e. radiolabelled  $[(86)\text{Y}] \text{Y}-(\text{CHX-A}''\text{-DTPA})_4\text{-cetuximab}$  for positron emission tomography (PET) and  $[(90)\text{Y}] \text{Y}-(\text{CHX-A}''\text{-DTPA})_4\text{-cetuximab}$  for radioimmunotherapy applied in combination with external beam radiotherapy, improvement of radiation response by radioimmunotherapy could be shown in tumours with higher expression of the EGFR and with high perfusion (Koi et al. 2014). These data support an important role of two simple factors, the target expression and the reachability of the target by the (in this case relatively large) molecules of the drug.

## 2.4 Non-EGFR family tyrosine kinases as therapeutic targets

Although the EGFR family of receptor tyrosine kinases (RTKs) have been the most widely studied as potential targets for tumour radiosensitization, the development of inhibitors against RTKs such as c-Met and IGF-1R has resulted in growing interest in the potential role of other RTKs. As discussed below, there is significant data showing that the overexpression of some of these RTKs is associated with adverse clinical outcomes following radiotherapy treatment and therefore representing prognostic biomarkers. It is currently unclear whether expression levels may also prove to be predictive markers of response to novel inhibitors of these RTKs when combined with RT.

### 3 c-Met

c-Met is a transmembrane RTK that is activated by the extracellular binding of its ligand, hepatocyte growth factor (HGF), leading to receptor dimerization and phosphorylation of internal tyrosine kinase domains (Ponzetto et al. 1994). Activation of the HGF/c-Met pathway has been linked to numerous biological changes including tumorigenesis, resistance to apoptosis and increased cell proliferation and motility (Maulik et al. 2002). The pathway can be aberrantly upregulated by multiple mechanisms including autocrine HGF/c-Met signalling and overexpression of c-Met and by activating mutations (Yi and Tsao 2000; Ma et al. 2008; Ma et al. 2003). Downstream signalling of c-Met involves both the PI3K/Akt and MAPK pathways and therefore shows significant crosstalk with EGFR activation (Organ and Tsao 2011).

Ionising radiation has been shown to induce c-Met expression and activation of tumour cells, resulting in a more aggressive phenotype such as increased cell invasiveness. De Bacco et al. have shown that c-Met inhibitors cause pronounced tumour radiosensitization both in vitro and in vivo (De Bacco et al. 2011) and several other groups have reported similar findings using different c-Met inhibitors (Welsh et al. 2009; Bhardwaj et al. 2012). The exact mechanism by which c-Met inhibition causes radiosensitization is currently unclear, but there is significant evidence linking c-Met inhibition to decreased DNA damage repair (Welsh et al. 2009; Fan et al. 1998; Fan et al. 2000).

There is growing evidence that c-Met activation is associated with an adverse prognosis in many different tumour types. c-Met overexpression has been shown to be associated with worse progression-free and overall survival when compared with patients with little or no c-Met expression in glioblastoma patients treated with surgical resection, followed by adjuvant radiotherapy or chemoradiotherapy (Kong et al. 2009). Increased c-Met copy number has also been associated with worse survival in surgically resected NSCLC (Cappuzzo et al. 2009). Recent data have shown that high c-MET gene expression significantly correlates also with locoregional tumour control after postoperative radiochemotherapy in HNSCC, where patients with high c-MET gene expression show a substantially lower tumour control rate compared to patients with low expression (Linge et al. 2016)

Since c-Met signalling is frequently upregulated in many cancers which are often treated with radical radiotherapy such as HNSCC and lung cancer, there is significant interest in combining c-Met inhibitors with radiotherapy treatment. c-Met is overexpressed in over 80 % of HNSCCs (Burtness et al. 2013). High expression of c-Met has been shown to be associated with reduced response rates, and worse local failure-free and overall survival rates in oropharyngeal cancer following radical radiotherapy (Aebersold et al. 2001).

Several inhibitors against c-met have been described. Since the side effect profiles of leading clinical candidates such as crizotinib (which also targets ALK) and tivantinib are becoming familiar, there will be significant interest in combining these agents with radiotherapy treatment (Ou 2011; Scagliotti et al. 2013). Once the toxicity associated with such combinations has been established, attempts can be made to identify biomarkers of response to these treatments including expression levels of c-Met.

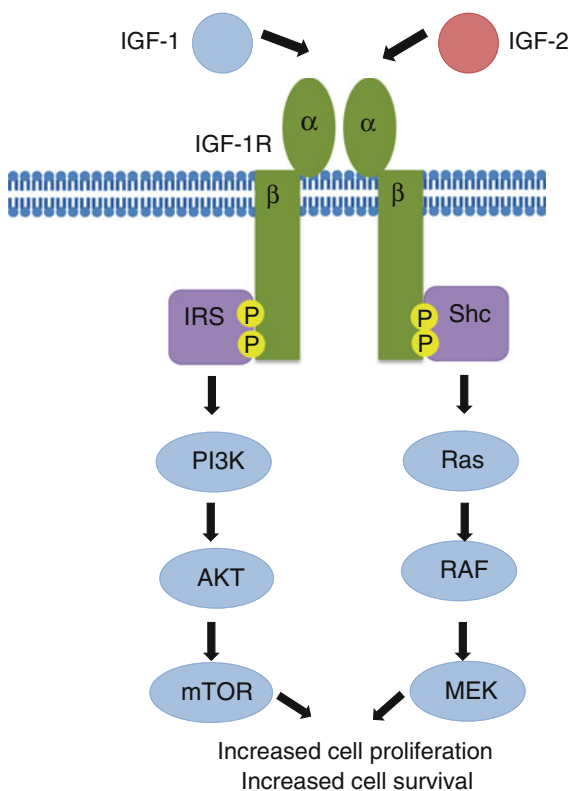


## 4 Insulin-Like Growth Factor 1 Receptor

The insulin-like growth factor 1 receptor (IGF-1R) is a cell membrane receptor that belongs to the IGF/insulin family of receptors which play important roles in tissue growth and development as well as overall metabolism. However, IGF-1R signalling also appears to play an important role in the transformation of cells, and cancer cell proliferation and metastasis (Arcaro 2013).

The IGF-1 receptor functions as a homodimer with two  $\alpha$  extracellular subunits containing a ligand-binding domain as well as two  $\beta$  transmembrane subunits which contain an intracellular tyrosine kinase component (Adams et al. 2000). IGF-1, IGF-2 and insulin interact with IGF-1R, although there is particularly high affinity between IGF-1 and IGF-1R. Ligand binding of IGF-1R results in autophosphorylation of tyrosine residues in the kinase domain and phosphorylation of the juxtamembrane tyrosines which act as a docking site for adaptor molecules such as Src-homology collagen protein (Shc) and the insulin receptor substrate (IRS) 1 and 2. This results in the activation of a complex downstream signalling cascade that includes the MAPK and PI3K/Akt pathways (Fig. 2) similar to EGFR activation (Favelyukis et al. 2001; Shelton et al. 2004; Zha and Lackner 2010).

**Fig. 2** Principal components of the IGF-1R pathway. IGF-1 and IGF-2 ligands bind to the extracellular domain of IGF-1R resulting in receptor cross-linking and autophosphorylation. Docking proteins such as IRS and Shc trigger activation of the PI3K-AKT and Ras-RAF pathways, respectively, leading to increased cell proliferation and resistance to cell death



The ultimate effects of IGF-1R activation include increased cell proliferation and resistance to chemotherapy- and radiotherapy-induced cell death (Arnaldez and Helman 2012).

Although no cancer-specific mutations of IGF-1R have been described, multiple studies have reported dysregulated IGF-1 signalling in many different malignancies. These include overexpression of the IGF-1 receptor in breast and colorectal cancer and the presence of elevated IGF-1 levels in many different tumour types including prostate, gastric, breast and colorectal cancer (Arcaro 2013).

There is an abundance of evidence linking aberrant IGF-1R signalling to radioresistance. Fibroblasts overexpressing IGF-1R have been shown to have increased radioresistance, while IGF-1R silencing has been shown to increase tumour radiosensitivity (Turner et al. 1997; Yavari et al. 2010; Rochester et al. 2005). In addition, the use of anti-IGF-1R antibodies has been shown to cause tumour radiosensitization both in vitro and in vivo (Riesterer et al. 2011; Iwasa et al. 2009).

Alterations in cell proliferation and DNA repair are the primary mechanisms by which disruption of IGF-1R signalling causes radiosensitization. IGF-1R is involved in regulating the DNA damage response pathway by its interaction with ataxia-telangiectasia-mutated (ATM) protein with IGF-1R silencing decreasing the activity of ATM and inducing tumour radiosensitization (Peretz et al. 2001; Macaulay et al. 2001). IGF-1R has been particularly implicated in DNA double-strand break (DSB) repair with inhibition of the IGF-1 receptor reducing DSB repair post-radiation as measured by the presence of Rad51 and  $\gamma$ H2AX (Iwasa et al. 2009). Although much work has been focused on the effects of IGF-1R on non-homologous end joining (NHEJ) by regulating Ku70/80, it has additionally recently been suggested that inhibition of IGF-1R also attenuates homologous recombination (Valenciano et al. 2012; Chitnis 2013).

The clinical effects of aberrant IGF1 signalling on patients treated with radiotherapy have been shown to be associated with adverse outcomes in many different tumours including breast (Turner et al. 1997); cervical (Lloret et al. 2008), and head and neck cancer (Lara et al. 2011). Data from a prospective head and neck cancer study showed that IGF-1R expression was associated with a 28.6-fold increased risk of treatment failure and supports the potential role of IGF-1R expression as a prognostic biomarker following radiotherapy (Moreno-Acosta et al. 2012).

Multiple monoclonal antibodies and small molecule inhibitors targeting IGF-1R have been described and have entered clinical trials (Arnaldez and Helman 2012). Figitumumab, a monoclonal antibody against IGF-1R, showed significant promise in a phase II study, but subsequent phase III studies in NSCLC with concurrent chemotherapy failed to demonstrate any benefit. Although it is possible that biomarkers could be developed and validated to identify subgroups of patients with metastatic disease who benefit from IGF1-R inhibition, the failure of these large phase III studies caused the development of several inhibitors to be discontinued (Yee 2012).

Disappointingly, even though the preclinical and clinical rationale for combining IGF-1R inhibitors with radiotherapy is compelling, there is a paucity of clinical studies in this area. Since there is ongoing interest in combining IGF-1R inhibitors

in the metastatic setting with additional inhibitors against such targets as EGFR and mTOR (Fidler et al. 2012), it seems justifiable to initiate clinical studies combining IGF-1R inhibitors with radiotherapy in patients with IGF-1R overexpression.

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## 5 Vascular Endothelial Growth Factor Receptor/Platelet-derived Growth Factor Receptor

As the vascular endothelial growth factor (VEGF) receptors and platelet-derived growth factor (PDGF) receptors have overlapping biological functions such as angiogenesis and are both inhibited by tyrosine kinase inhibitors such as sorafenib and sunitinib, which are already in clinical use, they will be considered together in this section (Homsí and Daud 2007).

The VEGF family is comprised of VEGF-A, -B, -C and -D and placental growth factor which can bind to three receptor tyrosine kinases (VEGFR-1, -2 and -3) (Olsson et al. 2006). The central role of VEGF-A binding to VEGFR-1 and VEGFR-2 to enable tumour angiogenesis has led to the development of several anti-VEGF treatments, of which the VEGF monoclonal antibody bevacizumab has been most widely used (Ferrara et al. 2004).

The PDGF family consists of PDGF-A, -B, -C and -D and exerts their effects by binding to the structurally similar PDGF transmembrane receptors (PDGFR- $\alpha$  and PDGFR- $\beta$ ). Ligand binding results in receptor dimerization and autophosphorylation of the PDGFR tyrosine kinase (Andrae et al. 2008). This propagates downstream signalling which, in common with EGFR, c-Met and IGF-1R, includes the MAPK and PI3K/Akt pathways known to be associated with increased cell proliferation and resistance to cell death.

PDGF has been implicated as a mediator of both acute and chronic inflammation and, in particular, has been associated with the development of pulmonary, hepatic, cardiac and renal fibrosis (Andrae et al. 2008). Preclinically, the expression levels of PDGF and PDGFR have been shown to be elevated in rats with radiation-induced pulmonary fibrosis (Tada et al. 2003). In vivo experiments have shown that three different drugs targeting the PDGF receptor tyrosine kinase reduced radiation-induced pulmonary fibrosis in irradiated mice on the basis of physiological, radiological and histological endpoints (Abdollahi et al. 2005). Since pulmonary pneumonitis and fibrosis are key dose-limiting factors in radiotherapy treatment for lung cancer, there is therefore potential interest in using PDGFR inhibitors as antifibrotic treatments to protect normal lung tissue from the side effects of radiotherapy (Gross 1977; Li et al. 2007).

PDGF is also known to contribute to tumour angiogenesis primarily through actions on pericytes and vascular smooth muscle cells (Battagay et al. 1994; Risau et al. 1992; Xue et al. 2012; Abramsson et al. 2003). Although it may seem surprising that disrupting tumour angiogenesis should augment the effects of radiotherapy, since disrupting the tumour vasculature could lead to reduced perfusion and therefore increased hypoxia, there is significant preclinical evidence that

antiangiogenesis treatments cause tumour radiosensitization. This has resulted in the development of over forty clinical studies combining radiation and angiogenesis inhibition. Although many of these studies are with bevacizumab, several studies are combining radiotherapy with the tyrosine kinase inhibitors, sorafenib and sunitinib, which both inhibit PDGFR as well as VEGFR (Kleibeuken et al. 2012).

It has been suggested that the mechanism by which bevacizumab augments radiotherapy treatment may be due to “vascular normalization” in which the normal structure and function of the tumour vessels are restored, thereby reducing tumour hypoxia, rather than by true inhibition of angiogenesis (Goel et al. 2011). Bevacizumab has been shown to cause such changes in murine xenograft experiments, but even in these murine models, the effects appear to be transient, and treatment beyond five days may potentially increase tumour hypoxia (Dings et al. 2007). The transient nature of this normalization may account for the disappointing results seen in two phase III trials showing that the addition of bevacizumab to chemoradiotherapy treatment for glioblastoma multiforme (GBM) failed to improve overall survival (Chinot et al. 2014; Gilbert et al. 2014)

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## 6 Conclusions

Although data are heterogeneous, EGFR expression itself plays a major role for prognosis of patients after radiotherapy. There is good evidence that the EGFR is involved in repopulation of tumour cells during fractionated radiotherapy, leading to worse locoregional tumour control rates for high EGFR-expressing head and neck squamous cell carcinoma as compared to lower EGFR-expressing tumours. Inhibition of the EGFR during radiotherapy leads to improvement of local tumour control in head and neck squamous cell carcinoma, when anti-EGFR antibodies (cetuximab) are used. However, the effects are heterogeneous and warrant the establishment of biomarkers to identify individuals likely to respond to combined cetuximab/RT treatment. For other ErbB inhibitors, similar functions may exist, but data are sparse and conclusions currently not justified.

The realization that non-EGFR receptor tyrosine kinases such as c-Met and IGF-1R may play important roles in tumour radioresistance, combined with the development of effective compounds against these targets, has prompted significant interest from within the radiation oncology community. There is clearly the potential for these RTKs to be used as biomarkers to predict response to radiotherapy treatment. In addition, future studies may ascertain whether patients with the activation of these pathways benefit from treatment adaptations such as radiation dose escalation. A potentially even more exciting approach is to combine radiotherapy with inhibitors against these RTKs, and this may significantly increase the effectiveness of radiotherapy.

Importantly, RTKs such as c-Met, IGF-1R and PDGFR all involve a degree of crosstalk with EGFR by signalling through the MAPK and PI3K/Akt pathways. Effective inhibitors against several downstream components of these pathways have been shown to cause tumour radiosensitization preclinically. These include MEK

inhibitors such as AZD6244 (Astrazeneca) (Chung et al. 2009) which is now being used in phase I studies with concurrent radiotherapy in NSCLC and chemoradiotherapy in rectal carcinoma, and PI3K inhibitors such as BKM120 (Novartis) (Fokas et al. 2012) which is in early phase trials with radiotherapy in NSCLC and chemoradiotherapy in GBM. It remains to be seen whether inhibiting these downstream components of several different classes of RTK may be the most effective and pragmatic approach to radiosensitizing tumours clinically rather than by inhibiting the RTKs directly.

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# Hypoxia as a Biomarker and for Personalized Radiation Oncology

Dirk Vordermark and Michael R. Horsman

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## Abstract

Tumor hypoxia is a clinically relevant cause of radiation resistance. Direct measurements of tumor oxygenation have been performed predominantly with the Eppendorf histograph and these have defined the reduced prognosis after radiotherapy in poorly oxygenated tumors, especially head-and-neck cancer, cervix cancer and sarcoma. Exogenous markers have been used for immunohistochemical detection of hypoxic tumor areas (pimonidazole) or for positron-emission tomography (PET) imaging (misonidazole). Overexpression of hypoxia-related proteins such as hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) has also been linked to poor prognosis after radiotherapy and such proteins are considered as potential endogenous hypoxia markers.

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## Keywords

Tumor hypoxia · Tumor oxygenation · Pimonidazole · Hypoxia-inducible factor-1 $\alpha$

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## 1 Introduction

Intratumoural hypoxia is a clinically relevant condition causing radiation resistance of tumour cells. The insufficient supply of fast-growing tumours with blood vessels and the pathologic structure of intratumoural vessels, respectively, have been

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associated with two mechanisms of tumour hypoxia. Specifically, these are diffusion-limited hypoxia caused by long distances of individual tumour cells from the nearest blood vessel versus perfusion-limited tumour hypoxia which can also occur close to (non-perfused) blood vessels due to the presence of vascular leaks, thrombosis or shunts. These two types have also been referred to as “chronic” and “acute” hypoxia, respectively. However, oxygenation of a tumour is considered a dynamic process, and in many tissues, intermittent changes of oxygen concentration are observed (“cyclic hypoxia”).

The classic explanation of hypoxia-associated radioresistance in tumour cells is the interference of molecular oxygen with DNA repair. Oxygen has been shown to bind to sites of radiation-induced DNA damage, thereby causing fixation of DNA lesions and preventing repair. In *in vitro* experiments, an oxygen-enhancement ratio (OER) of 2–3 has been determined, meaning that compared to a reference dose of ionizing radiation under normoxic conditions, 2–3 times this dose has to be given to achieve equivalent cell kill under anoxic (0 % oxygen) conditions.

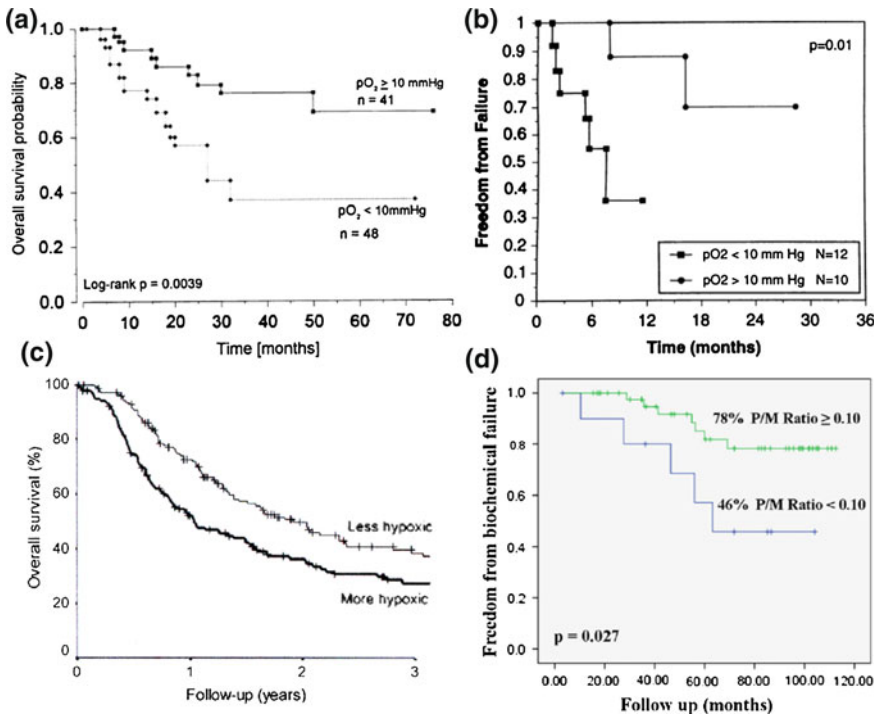
*In vivo*, an additional mechanism causing treatment resistance of hypoxic tumours is the selection of tumour cells with increased anti-apoptotic, proliferative and metastatic characteristics by conditions of hypoxia (Graeber et al. 1996). This chapter will discuss currently available methods to detect intratumoural hypoxia and their clinical applicability.

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## 2 Direct Measurements of Tumour Oxygenation

Probably, the most direct method for identifying hypoxia in tumours involves inserting electrodes into the tissue and monitoring the actual oxygenation status. This approach was first applied to human tumours in the 1960s using “home-made” glass electrodes. These early polarographic electrodes were, however, generally cumbersome and fragile, and only a few  $pO_2$  values 3–4 mm below the surface of the tumour were possible. Nevertheless, clinical data were obtained in cervix (Kolstad 1968) and head-and-neck (Gatenby et al. 1988) cancer patients that clearly demonstrated a relationship between the oxygenation measurements and outcome to radiation therapy, in that those patients with tumours that were better oxygenated had a significantly superior local response to irradiation.

This whole area was revolutionized with the development of the Eppendorf histograph, which had two distinct improvements. The first was having a more robust electrode with the oxygen sensor protected inside a metal needle. A second improvement was the attachment of this needle to a stepping motor that allowed for multiple measurements along the needle track through the tumour; thus, detailed oxygen partial pressure ( $pO_2$ ) distributions were possible. Numerous clinical studies were thus undertaken in a variety of human tumour types. The results clearly showed that hypoxia was a characteristic feature of virtually all human tumours investigated, although the degree of hypoxia could be variable (Vaupel et al. 1989). Probably, the most significant finding from these studies was the confirmation that hypoxia influenced outcome to therapy. This has been reported for head and neck



**Fig. 1** Relationship between tumour oxygenation estimated prior to therapy using the Eppendorf histograph and eventual outcome to that therapy. **a** Overall survival for 89 cervix patients given surgery or radiotherapy with curative intent in which the median  $pO_2$  values were above or below 10 mmHg. **b** Freedom from distant failure in 22 patients with soft tissue sarcomas receiving preoperative irradiation and hyperthermia in which the tumour median  $pO_2$  values were above or below 10 mmHg. **c** Overall survival for 397 head-and-neck cancer patients after primary radiation therapy in which the percentage of  $pO_2$  values less than or equal to 2.5 mmHg was above (more hypoxic) or below (less hypoxic) the median value of 19 %. **d** Freedom from biochemical failure for 57 prostate patients treated with brachytherapy in which the prostate/muscle (P/M) mean  $pO_2$  ratio was above or below 0.10. Composite figure derived from Hoeckel et al. (1996) (a); Brizel et al. (1996) (b); Nordmark et al. (2005) (c); and Turaka et al. (2011) (d)

(Nordmark et al. 1996, 2005; Brizel et al. 1997; Stadler et al. 1999; Rudat et al. 2001), cervix (Hoeckel et al. 1993, 1996; Knocke et al. 1999; Fyles et al. 1998, 2006; Lyng et al. 2000), soft tissue sarcomas (Brizel et al. 1996; Nordmark et al. 2001), and prostate (Turaka et al. 2011). Examples of the typical results obtained in each of these clinical sites are illustrated in Fig. 1 and clearly show that patients with more hypoxic tumours had a poorer outcome to therapy. Perhaps the most striking results were those made in cervix and sarcomas that showed hypoxia to influence outcome in patients in which surgery was the primary or only treatment (Hoeckel et al. 1996; Nordmark et al. 2001), suggesting that hypoxia could also influence malignant progression, especially metastatic spread. In fact, one other study in cervix was able to show that the primary tumours of patients with

metastases were indeed more poorly oxygenated than those of patients without metastases (Sundf r et al. 1998).

Today, the Eppendorf electrode is no longer commercially available, and there are a number of reasons for this. Without using concurrent imaging during the oxygen measurements, it was impossible to state whether the values obtained were from viable tissue, and even where this was done one could not state whether the cells in the hypoxic regions were clonogenic; the tumours themselves had generally to be easily accessible; and the technique was invasive. Furthermore, despite the positive findings between the Eppendorf measurements and treatment outcome, the machine was never predictive of response on a patient-to-patient basis. This was clearly illustrated in one of the first clinical studies using head-and-neck cancer patients (Nordsmark et al. 1996). Here, the 35 patients in which tumour oxygenation measurements were performed could be separated into two distinct groups with those patients having the more hypoxic tumours showing a significantly lower local tumour control following conventional radiation therapy. However, some 40 % of the patients did not fall within the correct category; they were hypoxic but controlled or had no hypoxia yet failed. Despite the various limitations, the results obtained from the Eppendorf studies must still be considered positive in that it supplied us with a tremendous level of information about tumour hypoxia and its importance.

Another approach that may have the potential to measure tumour oxygenation status involves the use of fibre optic probes. Unlike the Eppendorf histogram electrode, these do not consume oxygen with each measurement; thus, continuous observations of oxygenation status in the same tumour region is possible (Griffiths and Robinson 1999). Preclinical studies comparing the commercially available Oxford-Optronix OxyLite sensor with the polarographic techniques (Collingridge et al. 1997; Braun et al. 2001; Seddon et al. 2001; Wen et al. 2008), or classical paired survival curve estimates of radiobiological hypoxia (Urano et al. 2002), reported similarities and differences depending on the tissue type, tumour size or the changes observed using various modifiers of tumour hypoxia. Although fibre optic probes have the potential to not only measure the pretreatment level of tumour hypoxia, but also to monitor tumour oxygenation status continuously during and after treatment, there has as yet been no clinical application in cancer.

Other less invasive attempts to directly measure tumour oxygenation have involved phosphorescence tomography- or magnetic resonance (MR)-based approaches. The former requires the infusion of water-soluble phosphor probes into the vasculature (Vikram et al. 2007) and has been used to map oxygen concentration in preclinical tumour models (Wilson and Cerniglia 1992; Fukumura et al. 2001), but again has not been used in patients. The MR approaches include monitoring oxygen-sensitive reporter molecules ( $^{19}\text{F}$ -oximetry). Several such molecules have been developed including perfluorochemical emulsions and hexafluorobenzene (Pacheco-Torres et al. 2011). The latter approach allowed for actual quantification of the MR signals and conversion into oxygen concentrations at the pixel level (Zhao et al. 2005). However, systemic toxicity required the imaging agent to be injected directly into tumours limiting its potential clinical application. An alternative MR method is electron paramagnetic resonance (EPR),

which detects paramagnetic materials that have been injected into tissues (Krishna et al. 2012). It can provide quantitative and repeated 3D estimates of oxygenation and has been extensively used in preclinical studies and even in patients for a range of different clinical problems (Swartz et al. 2004). Although many of the preclinical studies have focused on tumour hypoxia, the clinical application of EPR in cancer has, however, been somewhat limited (Krishna et al. 2012).

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### 3 Exogenous Markers of Hypoxia

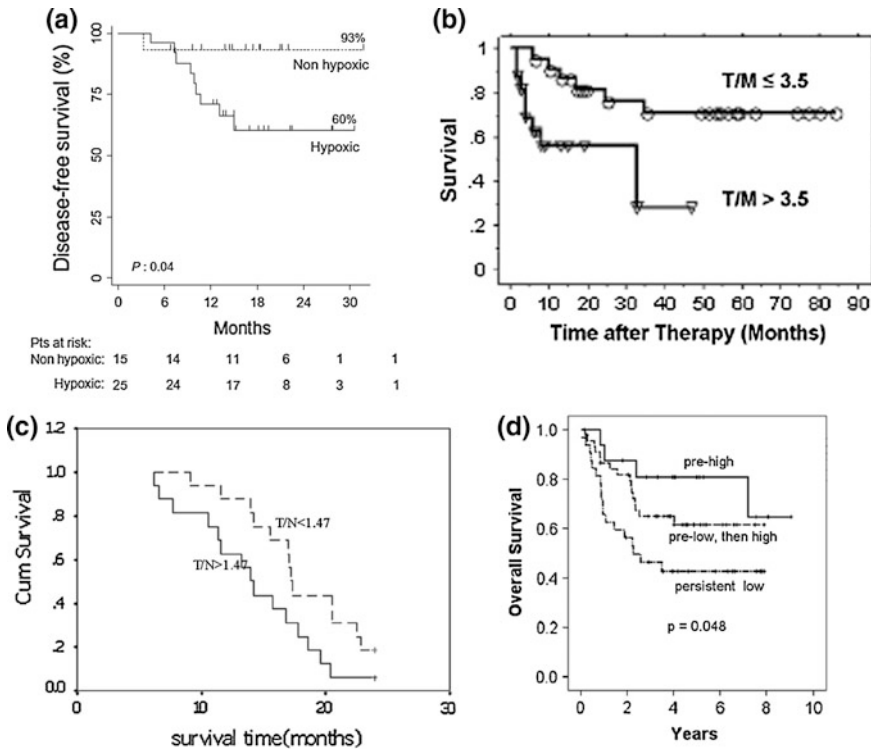
The oxygen mapping techniques described above involve injecting exogenous agents to directly ascertain oxygen values that are low and equivalent to hypoxia. A more widely studied method for indirectly detecting tumour hypoxia involves the administration of exogenous compounds that under hypoxic conditions undergoes a chemical change from a non-reactive species to a highly reactive product that then binds to macromolecules within the cell. Subsequent application of techniques that can identify this bound product will allow us to demonstrate the presence of hypoxia. The most popular agents used in this context have been 2-nitroimidazole-based markers. These nitroimidazole compounds were originally developed as hypoxic cell radiosensitizers, with the 2-nitroimidazoles being the most effective agents for enhancing radiation response in preclinical models (Adams and Cooke 1969). Such compounds are characterized by having an NO<sub>2</sub> grouping attached to the imidazole ring structure. This NO<sub>2</sub> group can undergo a 6-electron intracellular reduction to produce NH<sub>2</sub>, and although the NO<sub>2</sub> and NH<sub>2</sub> moieties are generally inactive, one of the formed intermediates is highly reactive and can bind to any macromolecule within the cell (Horsman et al. 2012). In the presence of oxygen, typically above 10 mmHg, the first electron reduction species formed reacts with oxygen and returns to the NO<sub>2</sub> moiety with the subsequent production of oxygen radicals that ultimately form hydrogen peroxide, and it is this lack of further reduction that gives rise to the hypoxia specificity. The bound product formed under hypoxia can be identified either using an antibody to the product or by labelling the original compound with a radioactive tracer such as <sup>1</sup>H or <sup>14</sup>C. The most commonly used nitroimidazole is pimonidazole, the binding of which in preclinical studies was found to correlate with radiobiological hypoxia (Raleigh et al. 1999). Additional clinical studies showed that the degree of pimonidazole binding was related to radiation-induced local tumour control in head-and-neck cancer (Kaanders et al. 2002), but not cervix (Nordsmark et al. 2006). Similar positive findings for head-and-neck cancer patients were reported between radiotherapy outcome and the degree of hypoxia estimated using another nitroimidazole marker, EF5 (Evans et al. 2007).

By labelling the nitroimidazole compound with <sup>18</sup>F will allow for the hypoxia produced bound product to be identified using positron emission tomography (PET). The first tracer developed for hypoxia PET imaging was a [<sup>18</sup>F]-fluorinated version of the radiosensitizer misonidazole (FMISO) and was found to be capable of identifying hypoxia in a range of human tumours (Rasey et al. 1996). It was followed by a group of compounds based on another radiosensitizer, etanidazole

(i.e. EF3/5). These markers have a relatively high lipophilicity which allows for easy penetration of cell membranes and diffusion into tumour tissue, but simultaneously limited the clearance of unbound tracer, thus leading to relatively low tumour-to-reference tissue ratios. Other fluorinated nitroimidazole compounds have been developed which are more water soluble than FMISO and therefore easier to clear from non-hypoxic tissue. These have included fluoroetanidazole (FETA), fluoroerythronitroimidazole (FETNIM), fluoroazomycin arabinofuranoside (FAZA) and HX4, of which the latter two are currently in clinical evaluation (Schuetz et al. 2010; van Loon et al. 2010). It is difficult to say whether one tracer is superior to another in identifying tumour hypoxia, since there has never been any systematic examination of all the 2-nitroimidazoles tracers in the same tumour model or patient population. The ideal tracer would be one in which clearance of unbound tracer is complete at the time of imaging; thus, only bound material indicative of hypoxia is measured. This can take many hours and even days to achieve, but such measurements have to take into account decay of the radioactive marker and normal clinical schedules. As a result, static scans are typically made 2–4 h after tracer administration, which results in low inter-tissue and intratumour contrast. An alternative approach involves labelling the nitroimidazoles with long-lived radionuclides, for example [ $^{124}\text{I}$ ]-iodoazomycin arabinoside ( $^{124}\text{I}$ -AZA) and [ $^{124}\text{I}$ ]-iodoazomycin galactoside ( $^{124}\text{I}$ -AZG), allowing for delayed scans up to 24 and 48 h after tracer administration. Unfortunately, the results have been disappointing with no improvement in image contrast and poor counting statistics (Rischi et al. 2006; Reischi et al. 2007), and it is unlikely that the problems inherent to hypoxia PET can be solved exclusively by better tracers. One small study in head-and-neck cancer patients (Thorwarth et al. 2005) demonstrated that pharmacokinetic analysis of the shape of tumour time activity curves (TACs) obtained from dynamic PET scans increased prognostic accuracy compared to traditional analysis of static PET images. However, dynamic scans are cumbersome and expensive, and cause inconvenience to patients; the analysis is complex; and different estimates of hypoxia can be obtained depending on the kinetic model and tumour type used.

Regardless of whether static or dynamic assessment is applied, one of the major issues with PET markers is that the cells must be hypoxic for significant time periods to be detected, which means that such markers are more likely to identify diffusion-limited chronic hypoxia rather than acute hypoxia resulting from transient fluctuations in blood flow (Horsman et al. 2012). Another significant problem facing the application of PET hypoxia markers is one of resolution in which the voxel sizes identified in the PET scans are much larger than most of the hypoxic structures (Horsman et al. 2012). Thus, the actual PET image does not accurately reflect the true hypoxia heterogeneity at the microregional level.

Several of the nitroimidazole-based PET markers have undergone clinical evaluation with respect to correlating the hypoxia estimates with outcome to radiation therapy. The majority of studies involved FMISO measurements in head-and-neck cancer patients (Rajendran et al. 2006; Rischi et al. 2006; Thorwarth et al. 2006; Eschmann et al. 2007; Dirix et al. 2009; Lee et al. 2009; Kikuchi et al. 2011;



**Fig. 2** Results from four different clinical trials showing the relationship between hypoxia imaging and outcome to therapy. **a** Disease-free survival in 40 head-and-neck cancer patients based on the preradiation therapy estimate of hypoxia as determined by a tumour-to-muscle ratio of  $\geq 1.4$  from  $[^{18}\text{F}]$  FAZA PET measurements. **b** Progression-free survival in 38 cervical cancer patients receiving radiotherapy and chemoradiotherapy in which the tumour/muscle (T/M) levels of  $^{60}\text{Cu}$ -ATSM were above or below the threshold dose of 3.5. **c** Overall survival for 32 non-small-cell lung cancer patients in which the tumour-to-normal tissue (T/N) ratio measured with  $^{99\text{m}}\text{Tc}$ -HL91 SPECT before radiation therapy was above/below 1.47. **d** Disease-specific survival in 98 cancer patients based on the level of perfusion measured with DCE-MRI either before or before and during radiation treatment. Composite figure derived from Mortensen et al. (2012) (a); Dehdashti et al. (2008) (b); Li et al. (2006) (c); and Mayr et al. (2010) (d)

Zips et al. 2012). Two other studies in head and neck used either FETNIM (Lehtio et al. 2004) or FAZA (Mortensen et al. 2012). Results from the latter study using FAZA as the imaging agent are illustrated in Fig. 2 and clearly show that like all the other head-and-neck studies, patients with hypoxic tumours had a significantly poorer outcome. Similar findings were found using FETNIM in lung (Li et al. 2010) and oesophagus (Yue et al. 2012), FAZA in sarcomas (Khamly et al. 2008) and cervix (Scheutz et al. 2010) and FMISO in central nervous system tumours (Spence et al. 2008). Despite the positive findings obtained with all these nitroimidazole-based hypoxia PET markers, the situation remains the same as seen with the oxygen electrode methods in that although we can verify the presence of hypoxia in human



tumours and demonstrate that it influences outcome to therapy, we still cannot use the results to select those patients that are hypoxic on an individual basis.

A chemically different group of putative PET markers that may have the potential to identify tumour hypoxia includes Cu-ATSM [Cu(II)-diacetyl-*bis*(*N*<sup>4</sup>-methylthiosemicarbazone)], which can be labelled with a variety of positron-emitting isotopes of copper. Cu-ATSM has been shown to have high membrane permeability and fast tumour uptake, thus allowing for rapid imaging after injection (Dearling et al. 1998), but its exact retention mechanism is still not completely understood. The hypoxia specificity is believed to occur because while the Cu<sup>2+</sup> moiety can easily pass across cell membranes, under low oxygen conditions the Cu<sup>2+</sup> is converted to Cu<sup>1+</sup> which is then trapped within cells (Vävere and Lewis 2007). A good correlation between Cu-ATSM and pO<sub>2</sub> measurements (Lewis and Welch 2001; O'Donoghue et al. 2005) and nitroimidazole-based hypoxia markers (O'Donoghue et al. 2005; Dence et al. 2008) have been shown in preclinical studies, although these effects are time- and tumour-dependent (O'Donoghue et al. 2005). Measurements in patients with lung (Dehdashti et al. 2003), cervix (Dehdashti et al. 2008), rectal (Dietz et al. 2008) or head-and-neck (Minagawa et al. 2011) cancer support the possibility of using Cu-ATSM as a marker of outcome to radiotherapy. The results from the cervix trial (Dehdashti et al. 2008) are shown in Fig. 2 and clearly demonstrate that those patients with a higher tumour uptake of Cu-ATSM, and presumably more hypoxic, had a lower progression-free survival. However, additional studies have shown Cu-ATSM to be affected by mechanisms other than hypoxia and that it is also insensitive to treatments that modify tumour oxygenation (Yuan et al. 2006); thus, its potential to be used as a specific marker for tumour hypoxia remains unclear.

The use of alternative radioactive labels allows for the possibility to use other non-invasive imaging techniques to identify hypoxia in tumours. Such an approach has been achieved with a number of [<sup>123</sup>I/<sup>125</sup>I]-iodoazomycin derivatives which can be detected using single-photon emission computer tomography (SPECT). Of these, only [<sup>123</sup>I]-iodoazomycin arabinoside (IAZA) has undergone clinical evaluation (Urtasun et al. 1996), and it was found that head-and-neck cancer patients with positive IAZA scans had a poorer outcome to radiotherapy than those patients with negative scans. Other potential SPECT markers for hypoxia that have been developed include <sup>99m</sup>Tc-labelled compounds, such as BMS 181321 and BRU59-21, and complex ligands, specifically HL91. BRU59-21 was investigated in a phase I study in patients with head-and-neck cancer, and a significant correlation was found with pimonidazole binding (Hoebbers et al. 2002). HL91 uptake was studied in non-small-cell lung cancer patients prior to radiation therapy, and the results, as shown in Fig. 2, demonstrated that those patients with the highest uptake had a significantly poorer response (Li et al. 2006). Various [<sup>19</sup>F]-labelled nitroimidazole compounds have also been developed which can be detected using MR. To-date, two [<sup>19</sup>F]-labelled nitroimidazoles have been developed, namely [<sup>19</sup>F]-EF5 and [<sup>19</sup>F]-SR 4554. Of these, only [<sup>19</sup>F]-SR 4554 underwent some clinical evaluations (Seddon et al. 2003), but there has not been any real follow-up.

An alternative MR approach that utilizes measurements made after injecting an exogenous marker is dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). This involves intravenously injecting a contrast agent and then monitoring its extravasation over several minutes from a region of interest (Nielsen et al. 2012). The focus with these estimates is actually on tumour perfusion, but this may still be an excellent method for identifying tumour hypoxia; oxygen delivery occurs via the vascular supply so the measurements could reflect chronic hypoxia, and changes in perfusion are clearly responsible for fluctuating hypoxia (Horsman et al. 2012). Clinical studies have been performed with DCE-MRI and reported that the parameters obtained actually correlated with oxygen electrode measurements in cervix (Cooper et al. 2000; Lyng et al. 2001), pimonidazole binding in head and neck (Newbold et al. 2009; Donaldson et al. 2011) and [ $^{18}\text{F}$ ]-FMISO uptake in glioblastoma multiforme (Swanson et al. 2009) and head-and-neck nodal metastases (Jansen et al. 2010). Several studies have also attempted to correlate the DCE-MRI measurements with radiotherapy outcome in patients with cervical cancer (Loncaster et al. 2002; Mayr et al. 2010; Andersen et al. 2012) and reported that those patients with supposedly more hypoxic tumours had a poorer response to radiotherapy, as illustrated in Fig. 2. Tumour perfusion can also be estimated with PET following administration of [ $^{15}\text{O}$ ]-labelled water. However, the clinical studies that used this method in head-and-neck cancer have produced conflicting results. One study reported that the [ $^{15}\text{O}$ ]-labelled water perfusion estimates correlated reasonably well with [ $^{18}\text{F}$ ]-EF5 measurements of hypoxia (Komar et al. 2008), but another study found that poor local tumour control and survival after radiation therapy were associated with high blood perfusion rather than the low perfusion one would expect to be indicative of hypoxia (Lehtio et al. 2004).

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## 4 Endogenous Markers of Hypoxia

As an alternative to invasive electrode measurements or exogenously applied hypoxia markers, molecules expressed under (patho)physiological conditions of low oxygenation have been studied as potential “endogenous” or “intrinsic” hypoxia markers. Most of these studies are related to hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), a transcription factor subunit considered to be the main regulator of the hypoxia response in mammalian cells. HIF-1 $\alpha$  protein accumulates in the nucleus under hypoxic conditions and binds to hypoxia-responsive elements in the promoter regions of hypoxia-regulated genes, including erythropoietin (EPO), carbonic anhydrase IX (CA IX), glucose transporter 1 (GLUT1) and vascular endothelial growth factor (VEGF). Therefore, both HIF-1 $\alpha$  protein itself and mRNA or protein of HIF-1-regulated genes could serve as indicators of tumour hypoxia.

An appropriate marker of hypoxic radioresistance should become overexpressed or accumulate at a level of hypoxia which is relevant for cellular radiosensitivity. The half-maximal oxygen effect on radiosensitivity is observed at about 0.5 %  $\text{O}_2$  (Hall 1988). HIF-1 $\alpha$  protein has been shown to be detectable in HeLa cells after 4 h at 20 %  $\text{O}_2$  with a moderate increase between 20 and 6 %, a strong increase below

6 % and a maximum expression at 0.5 % (Jiang et al. 1996). In U87 MG human glioma cells, a constant increase in HIF-1 $\alpha$  protein could be shown between 2 and <0.02 % O<sub>2</sub>, for *in vitro* hypoxia durations between 1 and 18 h (Vordermark and Brown 2003).

The temporal response of HIF-1 $\alpha$  protein response to hypoxia (and reoxygenation) has been described as rather rapid: in HeLa cells, induction of HIF-1 $\alpha$  protein was observed after 2 min of hypoxia, a maximum expression was seen after 1 h, and after 32 min of reoxygenation, the protein was again undetectable (Jewell et al. 2001). These data indicate that HIF-1 $\alpha$  protein expression in tissue can occur already at higher oxygen concentrations where radiosensitivity of cells is not yet compromised and that care must be taken in processing of surgical/biopsy tissue to account for the immediate response to changes in oxygenation. Colocalization studies of HIF-1 $\alpha$  and injectable hypoxia markers in xenograft tumours have supported the assumption that HIF-1-related hypoxia markers accumulate at higher O<sub>2</sub> concentrations (i.e. closer to perfused blood vessels) than exogenous markers such as EF5 (Vukovic et al. 2001).

Among the HIF-1-regulated genes, the membrane enzyme carbonic anhydrase IX (CA IX) has received the most attention as a potential endogenous hypoxia marker for use in radiotherapy. *In vitro*, a continuous increase of CA IX protein expression has been described in A-549 lung carcinoma cells exposed to decreasing O<sub>2</sub> concentrations from 5 to 0.1 % (Wykoff et al. 2000). Long-term hypoxia (24 h at 1.5 % O<sub>2</sub>) was found to result in higher CA IX levels than exposure for up to 10 h, suggesting that CA IX indicates predominantly prolonged exposure of cells to hypoxia (Lal et al. 2001). In FaDu head-and-neck squamous cell carcinoma, expression of CA IX increased over a radiosensitivity-relevant range of oxygen concentrations, resulting in a correlation of CA IX protein and cellular radioresistance (Vordermark et al. 2005).

The possibility to detect HIF-1 $\alpha$  protein and related proteins such as CA IX in archival tumour material from patients with an already known clinical course of disease has motivated researchers to analyse the relationship of marker expression on immunohistochemistry and clinical outcome (e.g. survival and local control) after cancer treatment, especially radiotherapy. In early histopathological series investigating a number of different tumour types, HIF-1 $\alpha$  has been detected in 40–82 % of prostate cancers, 80–100 % of colon adenocarcinomas and 29–83 % of breast adenocarcinomas (Zhong et al. 1999; Talks et al. 2000), with respective corresponding numbers for CA IX protein of 0, 100 and 26 % (Ivanov et al. 2001).

The HIF-1 $\alpha$  immunohistochemical staining pattern observed in sections of human tumour material is not consistent across tumour types and individual studies. In oropharyngeal carcinoma, a typical staining pattern of “diffusion-limited hypoxia” (i.e. positive cells in ring shape at a distance from a central blood vessel) was described in 65 % of positive tumours, with more diffuse patterns in the remainder (Aebersold et al. 2001). Other authors characterized the area of HIF-1 $\alpha$ -positive cells as close to a blood vessel (compatible with perfusion-related or “acute” hypoxia) versus distant from a vessel versus unspecific pattern (Haugland et al. 2002). A so-called perinecrotic staining pattern (i.e. accumulation of the

marker in the zone most distant from a blood vessel, near regions of necrosis) was also reported in studies of CA IX expression in cervical carcinoma, head-and-neck cancer and non-small-cell lung cancer (Beasley et al. 2001; Giatromanolaki et al. 2001; Loncaster et al. 2001). The use of the term “endogenous hypoxia marker” has been criticized, since immunohistochemical studies of pO<sub>2</sub> electrode measurement tracks in cervix cancer tumour tissue have shown no direct correlation of overexpression of the proteins HIF-1 $\alpha$ , CA IX or GLUT1 with the corresponding pO<sub>2</sub> reading (Mayer et al. 2006). This suggests that *in vivo* additional mechanisms other than the mere oxygenation level modulate expression of the putative endogenous hypoxia markers and that their expression is at least not hypoxia-specific.

However, a vast body of clinical literature suggests that a high level of expression of HIF-1-related proteins is related to poor outcome after cancer treatment. In head-and-neck cancer, several groups reported an association of HIF-1 $\alpha$  overexpression and reduced overall survival or disease-specific survival following surgery or radiotherapy or combination treatment (Aebersold et al. 2001; Beasley et al. 2002; Winter et al. 2006). The association of high CA IX expression with poor prognosis in head-and-neck cancer was seen to a lesser extent in comparable studies, and some groups found this marker to be prognostic only in combination with other potential hypoxia markers (Hui et al. 2002; De Schutter et al. 2005) or not at all (Eriksen and Overgaard 2007; Nordmark et al. 2007).

Cervix cancer, the other tumour entity with strong evidence from oxygen electrode studies of a relationship between tumour oxygenation and clinical response to radiotherapy, has been studied extensively regarding endogenous hypoxia marker expression. In three of the largest series treated with radiotherapy and/or surgery, HIF-1 $\alpha$  expression was also significantly associated with overall survival or disease-specific survival on multivariate analysis (Birner et al. 2000; Bachtary et al. 2003; Burri et al. 2003). Similar associations were found in some, but not all studies of CA IX expression in cervix cancer (Loncaster et al. 2001; Lee et al. 2007).

Other cancer types have been studied extensively regarding endogenous hypoxia marker expression, but with a stronger therapeutic focus on surgery and less impact of radiotherapy, among them breast cancer and lung cancer. Predominant associations of high marker expression and poor survival were observed here as well (review in Bache et al. 2008).

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## 5 Plasma Hypoxia Markers

In theory, the measurement of a hypoxia-related protein secreted from hypoxic tumour cells into the plasma could permit an integrated assessment of both the total tumour burden (“number of cells”) and their oxygenation level (“percentage of hypoxic tumour cells”). The best-studied secreted hypoxia-related protein is osteopontin (OPN), a tumour-associated glycoprotein secreted into bodily fluids and in the plasma of tumour patients. Plasma OPN level was shown by Le et al. (2003) to correspond with Eppendorf electrode measurements of tumour oxygenation in patients with head-and-neck cancer, suggesting a role for OPN as an

endogenous marker of tumour hypoxia. In a landmark study, Overgaard et al. (2005) showed that only patients with high plasma levels of OPN (upper tertile) significantly benefitted from the addition of nimorazole, a hypoxic radiosensitizer, compared to standard radiotherapy in patients with head-and-neck cancer. OPN may therefore serve as a marker by which to select head-and-neck cancer patients for intensified, hypoxia-specific, treatment. A molecular mechanism for the intracellular accumulation of OPN under hypoxia has been described (Sorensen et al. 2005; Zhu et al. 2005), although secretion of OPN may require additional steps (Said et al. 2005; Lukacova et al. 2006).

Elevated plasma or serum levels of OPN have been reported for several human cancer types including pancreatic, hepatocellular, colon, breast, prostate and lung cancer (Fedarko et al. 2001; Koopmann et al. 2004; Zhang et al. 2006). An association of high OPN plasma levels with poor prognosis has been established for different clinical situations. For instance, Isa et al. (2009) demonstrated that low plasma OPN measured before treatment correlated with improved overall survival and progression-free interval after chemotherapy for non-small-cell lung cancer. Mack et al. (2008) reported an association of elevated OPN plasma levels and inferior overall survival after carboplatin-/paclitaxel-based chemotherapy for advanced non-small-cell lung cancer. Blasberg et al. (2010) could show that OPN plasma levels significantly decreased after resection of early stage lung cancer and increased with later relapse, supporting the potential value of OPN as a biomarker for monitoring the treatment response in lung cancer. Recently, a pilot study of plasma hypoxia markers has also suggested a prognostic role of osteopontin response to radiotherapy of locally advanced non-small-cell lung cancer (Ostheimer et al. 2013)

Given the strong association with both electrode-measured oxygenation (Le et al. 2006) and response of tumour to surgery or chemotherapy in non-small-cell lung cancer, the prognostic potential of plasma OPN in radiotherapy of non-small-cell lung cancer was recently studied. Ostheimer et al. (2013) found in a pilot study of 55 patients with locoregionally advanced non-small-cell lung cancer that a panel of plasma biomarkers (OPN, CA IX and VEGF) in combination was an independent prognostic factor for overall survival in multivariate analysis. Early data on repeated measurements of OPN suggest a prognostic value of increasing plasma OPN over time in patients undergoing radiotherapy. In malignant glioma patients, OPN plasma levels did not decrease after surgical resection which may in part be explained by the fact that resection of such tumours is microscopically incomplete by nature, but also suggests that tumour-unrelated factors, such as wound healing, may contribute to total plasma osteopontin levels (Güttler et al. 2013). Nevertheless, a low plasma level of osteopontin at the end of postoperative radiotherapy identified patients with significantly improved overall survival in this pilot study. In patients with head-and-neck cancer, pretreatment OPN plasma levels were evaluated for outcome after surgery, radiotherapy, combined chemoradiation or sequential combinations. An adverse effect of high pretreatment OPN levels on survival and tumour control was confirmed for the different treatment arms (Petrik et al. 2006). In a separate series, lower pretreatment OPN levels were in favour of better tumour response and superior survival in head-and-neck cancer patients after radiotherapy alone (Snitcovsky et al. 2009).

## 6 Conclusions and Future Perspectives

Hypoxia is a characteristic feature of human tumours that has a major negative influence on determining tumour response to conventional therapy and is also an important factor in influencing malignant progression, both in terms of the aggressive growth of the primary tumour and its ability for metastatic spread. What is now needed is a method that allows us to identify those individual patients that have tumours containing significant levels of hypoxia, so that we can predict their outcome to therapy and where necessary select alternative treatments to eliminate that hypoxia.

Of course, one of the problems here is that hypoxia is often considered as a single entity when in fact we know that the degree and type of hypoxia found in tumours are very heterogenous (Horsman et al. 2012). At the very least, we have chronic and acute hypoxia, and it is not known whether both types respond equally well to therapy. Even if we could identify regions of both chronic and acute hypoxia within tumours, it is often difficult to state whether the cells in those hostile environments are actually viable and clonogenic. The situation becomes even more complicated because we also know that other microenvironmental factors such as intermediate hypoxia, low pH and glucose deprivation can influence malignant progression and thus outcome. The ideal imaging method must be able to identify all the critical factors. Furthermore, it must also give accurate, reliable and reproducible measurements, be easy to use on a routine basis and be applicable to any tumour type regardless of location. A non-invasive method would also be preferable. Clearly, no one technique that is currently available can achieve all these criteria. We must, therefore, either rely on measurements made with the best method there is or begin to combine modalities that can give us a better indication of the relevant parameters.

Once we have decided on the most relevant technique for imaging hypoxia, then there is the question of how do we deal with that hypoxia? Numerous preclinical studies have demonstrated a variety of methods that can be applied to eliminate hypoxia (Horsman et al. 2011), and many of these have been successfully applied in the clinic (Horsman and Overgaard 2007; Overgaard 2007), but none is currently in routine clinical use. It has also been suggested that in situations where we can actually image the distribution of hypoxia in tumours, we can then use that information to increase the radiation dose delivered to the tumour (Ling et al. 2000; Søvik et al. 2009; Bentzen and Gregoire 2011). But, whether that should be an increase to the gross tumour volume in which a substantial hypoxic volume has been identified or simply increase the dose to the biological target volume defined by the hypoxic area is not clear.

At present, we have a wealth of information about tumour hypoxia from a variety of clinically relevant techniques. Although we are not yet in a position to use that data to help us predict outcome on an individual patient basis or decide what we should do to tackle the hypoxia problem, the amount of effort being applied to this area would suggest that it is only a matter of time before we achieve those goals and the hypoxia problem eventually becomes obsolete.

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# Human Papilloma Virus as a Biomarker for Personalized Head and Neck Cancer Radiotherapy

Jesper Grau Eriksen and Pernille Lassen

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## Abstract

A dramatic increase in the incidence of HPV-related oropharyngeal cancer has been reported in some parts of the western world over the past 30 years. They constitute a clinically distinct subgroup of cancers in terms of molecular biology, patient characteristics, and treatment outcome. This chapter describes the molecular characteristics, epidemiology, and demographics of the HPV-related head and neck cancers and discuss available methods to detect HPV-related tumours. The impact of HPV-related biomarkers in clinical studies on radiotherapy only, altered fractionation, modulation of hypoxia, and concurrent chemo- or bio-radiotherapy are reviewed as well as the perspectives of de-escalation and immune-modulation are discussed.

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## Keywords

Head and neck cancer · Radiotherapy · HPV · P16 · Hypoxia · De-intensification

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## 1 Introduction

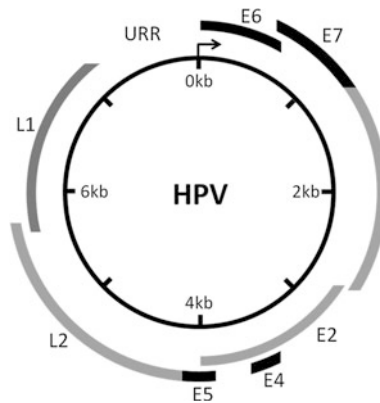
A causal relationship between human papillomavirus (HPV) and cervical cancer was established almost 40 years ago (Zur Hausen 1994) and HPV is necessary for the development of cervical cancers. Tobacco and alcohol were, until recently, considered to be the major risk factors in carcinogenesis of head and neck squamous cell carcinoma (HNSCC), but the putative role of HPV in HNSCC has been investigated since the 1980s, and at present sufficient molecular and pathological evidence exists to etiologically link HPV to a subset of HNSCCs, especially tumours arising in the oropharynx (OPSCC) (Gillison et al. 2000, 2012; Sudhoff et al. 2011). During the past 30 years, a rapid increase in the incidence of HPV-associated OPSCC has been reported in several Western countries while the incidence of HNSCC overall has declined (Chaturvedi et al. 2008; Hammarstedt et al. 2007; Lassen 2010; Mehanna et al. 2013). HPV DNA has been found in HNSCC from all sites (Kreimer et al. 2005) with a significantly higher prevalence in OPSCC compared to tumours arising in non-oropharyngeal sites. Moreover, HPV 16 is the dominant type in HNSCC, accounting for about 90 % of HPV DNA-positive HNSCCs, whereas HPV 18, 31, 33, and 35 are found in most of the remaining cases.

HPV-related HNSCC constitutes a clinically distinct subgroup of cancers in terms of molecular biology, patient characteristics (demographic and risk factor profile) and treatment outcome, and this on the whole differentiates it markedly from HPV-negative tumours, that typically have a strong association with tobacco. Tumour HPV status has proven to be the single strongest prognostic factor for outcome in radiotherapy of HNSCC, and separate therapeutic treatment strategies based on tumour HPV status are in the pipeline. The aim of this chapter is to summarize the current knowledge and understanding of HPV in HNSCC, with specific focus on the influence of HPV on radiotherapy outcome, including an assessment of the radiobiological modifications of head and neck cancer radiotherapy.

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## 2 HPV-Induced Oncogenesis

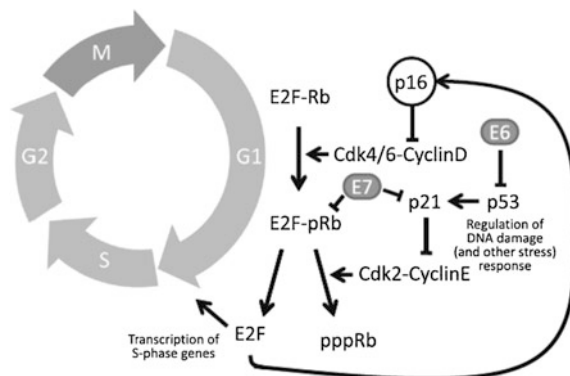
HPVs are strictly epitheliotropic and depend on epithelial differentiation for completion of their life cycle. Infection is established when the virus gains access to the basal cells of the skin or mucosal epithelium through micro-abrasions on the surfaces. Viral particles are then formed progressively as the basal cells differentiate and are eventually released when the cells reach the outer layer of the epithelium (Doorbar 1979; Doorbar 2005; Zur Hausen 2002). Currently, almost 150 different HPV types are isolated of which 120 types are fully sequenced. Based on their malignant potential, the HPVs are further subdivided into high-risk and low-risk types. HPVs are small double-stranded, circular DNA viruses with a genome of approximately 8,000 base pairs. The coding sequences have been classified as early



**Fig. 1** The HPV genome

(E) containing the early genes E1, E2, E4, E5, E6 and E7 and late (L) containing the late genes encoding the major (L1) and minor (L2) capsid proteins (Fig. 1). The presence of E3 and E8 has been recently described in only a few HPV types, but their function is yet unknown. Viral integration into the host cell genome occurs downstream the E6 and E7, often in the E1 or E2 region. However, in HNSCCs that can be attributed to HPV the viral genome is not exclusively integrated into the host genome but can also be found in episomal (extrachromosomal) form or a mixture of both similar to what is observed in cervical cancer samples. The E5 protein seems to be important in the early phase of infection, but in case of viral integration into host cell DNA the coding sequence of E5 is often deleted (Doorbar 2005). The molecular pathways in HPV-induced oncogenesis have been well described and the E6 and E7 oncoproteins play a key role in malignant transformation and maintenance, abrogating p53 and pRb tumour suppressor functions, respectively. The E6 oncoprotein disrupts the p53 pathway by ubiquitination and subsequent degradation of the p53 protein, which leads to uncontrolled cell cycle progression caused by lack of control at the G1/S and G2/M checkpoints (Nevins 2001). As a consequence of feedback loops, the functional inactivation of Rb by the E7 oncoprotein results in upregulation of the p16-protein. p16, encoded by the CDKN2A tumour suppressor gene, regulates the activity of CyclinD-CDK4/6 complexes (CDKs). When not inhibited by p16, these CDKs phosphorylate Rb leading to the release of the transcription factor E2F, which initiates cell cycle progression (Khleif 1996). In contrast, the bound Rb-E2F protein acts as a negative regulator, inhibiting transcription of several genes including CDKN2A. The functional inactivation of Rb by E7 therefore results in the release of the p16 gene from its transcriptional inhibition, by loss of negative feedback, and high expression levels of p16 is a characteristic feature of HPV-positive tumours (Khleif 1996; Chung and Gillison 2009; Smeets et al. 2007) (Fig. 2).





**Fig. 2** HPV E6 and E7 oncoproteins disrupt p53 and pRb regulatory pathways that govern the cell division cycle. The functional inactivation of pRb by E7 leads to upregulation of p16 as a consequence of feedback loops. With permission from Lassen P.

Genetic alterations caused by the actions of HPV oncoproteins E6 and E7 differ from the molecular profiles observed in HPV E6/E7 mRNA-negative HNSCC besides the upregulation of p16. Based on the current evidence, apparently HPV-induced carcinogenesis involves significantly fewer genomic alterations/accumulation of mutations than oncogenesis independent of HPV (Braakhuis et al. 2004; Slebos et al. 2006; Stransky et al. 2011). Particularly mutations in the tumour suppressor gene TP53 are less common in HPV-positive HNSCC compared to HPV-negative tumours (Gillison et al. 2000; Braakhuis et al. 2004) and also chromosomal aberrations of 3p, 9p and 17p are less frequent in HPV-related HNSCC (Braakhuis et al. 2004; Smeets et al. 2006). Thus, as a consequence of the specific HPV-induced carcinogenesis, primarily exerted by the E6 and E7 oncoproteins, HPV-positive tumours are characterized by a unique genotype and a molecular biological profile that distinguishes them from their virus-negative counterparts.

### 3 Epidemiology

A dramatic increase in the incidence of oropharyngeal cancer has been reported in several Western countries over the past 30 years (Chaturvedi et al. 2008; Hammarstedt et al. 2007; Lassen 2010; Marur et al. 2010; Blomberg et al. 2011). Based on the observations that, simultaneously, there has been an increase in the frequency of HPV-positivity among OPSCCs infection with HPV seems to be the dominant cause of this development. Moreover, in the same time period a decrease in tobacco-smoking seems to be responsible for a reduction in the incidence of HNSCC outside the oropharynx at least in Western countries. As a result, these opposite incidence trends are presently changing the traditional epidemiological pattern in HNSCC and will continue to do so in the near future, and it is estimated

that in countries with well-established, efficient screening-programs against cervical cancer, the incidence of HPV-related OPSCC will exceed that of cervical cancer by the year 2020 (Chaturvedi et al. 2011).

In 2007, the International Agency of Research of Cancer (IARC) declared that there is sufficient molecular and pathological evidence to etiologically link HPV 16 to a subset of HNSCCs, especially OPSCC. However, the natural history of oral high-risk HPV infection still remains to be fully elucidated and this is complicated by the fact that dysplasia is almost never detected in this region. Consequently, no efficient screening method exists to detect precancerous lesions in the oropharyngeal region. Although the exact mechanism is not known, oral–genital contact is assumed to be the primary mode by which HPV is transmitted to the oral mucosa, and several case control studies have shown an association between HPV-related HNSCC and sexual behaviour, most consistently the lifetime number of genital and oral sexual partners (Gillison et al. 2012).

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#### **4 Demography and Clinical Profile of HPV-Related HNSCC**

HPV-related HNSCCs frequently present with smaller primary tumours (T1–2), but more advanced nodal stage (Lassen et al. 2011; Rischin et al. 2010) resulting in the majority of patients with HPV-positive tumours being diagnosed at overall advanced clinical stages (American Joint Committee on Cancer, AACJ): Stage III–IV. The anatomical AJCC stage has traditionally been a reasonable prognostic indicator in HNSCC and is incorporated into treatment guidelines all over the world. However, based on the consistent findings from clinical trials that HPV-related disease has a favourable prognosis despite advanced stage at time of diagnosis, a revision of the AJCC stage and its prognostic implication is currently under debate.

Patients diagnosed with HPV-related HNSCC tend to be younger, have better performance status and less comorbidity and also a higher socioeconomic status compared with HPV-negative patients (Gillison et al. 2008; Smith et al. 2004; Worden et al. 2008). A history of heavy smoking (defined by >10 pack-years) is significantly less common among patients with HPV-related HNSCC than HPV-negative HNSCC although many patients with HPV-related disease do have a history of smoking (Chaturvedi et al. 2008; Gillison et al. 2008; Ang et al. 2010). Based on observations from the USA, there also seems to be quite a striking difference in the frequency of HPV-positivity between white and black patients with about 30 % of whites' tumours being HPV-positive compared with only 0–4 % of blacks' tumours (Settle et al. 2009). Moreover, the incidence of OPSCC has increased in whites but has decreased in blacks (Jemal et al. 2013) despite an apparent higher prevalence of oral HPV infections in blacks than whites (Gillison et al. 2012).

The characteristic microscopic appearance of HPV-related HNSCC, especially OPSCC, is another parameter separating this disease entity from the classical HNSCC, which has traditionally been grouped into poorly, moderately or highly

differentiated squamous cell carcinomas. The apparent predilection for HPV to target the highly specialized lymphoepithelium lining the lingual or palatine tonsillar crypts leads to a morphological resemblance between the tumours and this unique crypt epithelium. Consequently, HPV-related HNSCC tends to demonstrate non-keratinizing or only partially keratinizing morphology, with characteristic secondary features consisting of central necrosis with cystic change, tumour infiltrating lymphocytes and basaloid features, and very often surface dysplasia and prominent stromal desmoplasia are absent (Westra 2012). Based on these characteristic histopathological features of HPV-related HNSCC, it has been recommended by head and neck pathologists that the historical nomenclature of poorly, moderately or highly differentiated squamous cell carcinomas should be avoided and instead replaced by “HPV-related squamous cell carcinoma”. Moreover, incorporation of the typical microscopic appearance of an HPV-related tumour into the interpretation of various HPV detection assays (e.g. p16 immunohistochemical staining) is considered to be of significant importance and may ultimately lead to a more reliable and reproducible correlation with HPV status in HNSCC as described and recommended by El-Naggar and Westra (2012).

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## 5 HPV Detection Methods

The reported prevalence rates of HPV DNA in head and neck cancer specimens show considerable variation (Kreimer et al. 2005; Braakhuis et al. 2004) which in part can be explained by tumour site differences, i.e. viral prevalence is relatively low in the oral cavity and high in the tonsil (Gillison et al. 2000; Kreimer et al. 2005; Isayeva et al. 2012; Lingen et al. 2013). Another important contributing factor to the observed differences in HPV prevalence can be ascribed to the use of different detection methods. The most widely applied detection methods are based on PCR amplification of viral DNA. Generally, these assays have a high sensitivity, enabling detection of very few DNA copies per sample. However, this might yield false-positive results, due to sample to sample contaminations or detection of transient infections in which the virus is transcriptionally inactive, and as such the specificity of the methods is reduced (Smeets et al. 2007). Measuring levels of E6 and E7 mRNA by quantitative real-time PCR (qRT-PCR) assays increase specificity (Smeets et al. 2007; D’Souza et al. 2007), but fresh frozen tissue material is required for this method (Braakhuis et al. 2004; Smeets et al. 2006) which limits the present clinical applicability. Another commonly used method is the type-specific HPV DNA detection by in situ hybridization (ISH) assays. These assays are capable of detecting multiple HPV subtypes, their sensitivity is somewhat lower than the PCR-based assays, but on the other hand, they allow for visual confirmation of HPV DNA within individual tumour cell nuclei (Chung and Gillison 2009; Begum et al. 2005). Immunohistochemical (IHC) staining of tumour p16 expression has gained broad acceptance as a biomarker of infection with HPV in HNSCC, and a high correlation between HPV and p16 expression in HNSCC, particularly oropharyngeal carcinomas, has consistently been reported (Smeets et al. 2007; Shi

et al. 2009; Licitra et al. 2006; Lassen et al. 2009; Weinberger et al. 2006). The method is relatively standardized and easily applicable on formalin-fixed paraffin-embedded (FFPE) samples, which makes implementation of the method in the daily clinical routine feasible (Lewis 2012). Moreover, since transcription of E7 precedes upregulation of p16, it has been suggested that p16 positivity may identify specifically those HPV infections that are biologically relevant in carcinogenesis. Expression of p16 is not limited to HPV-positive tumours though, and using this marker alone as an indicator of biologically relevant HPV infections inevitably entails the risk of including some virally false-positive results.

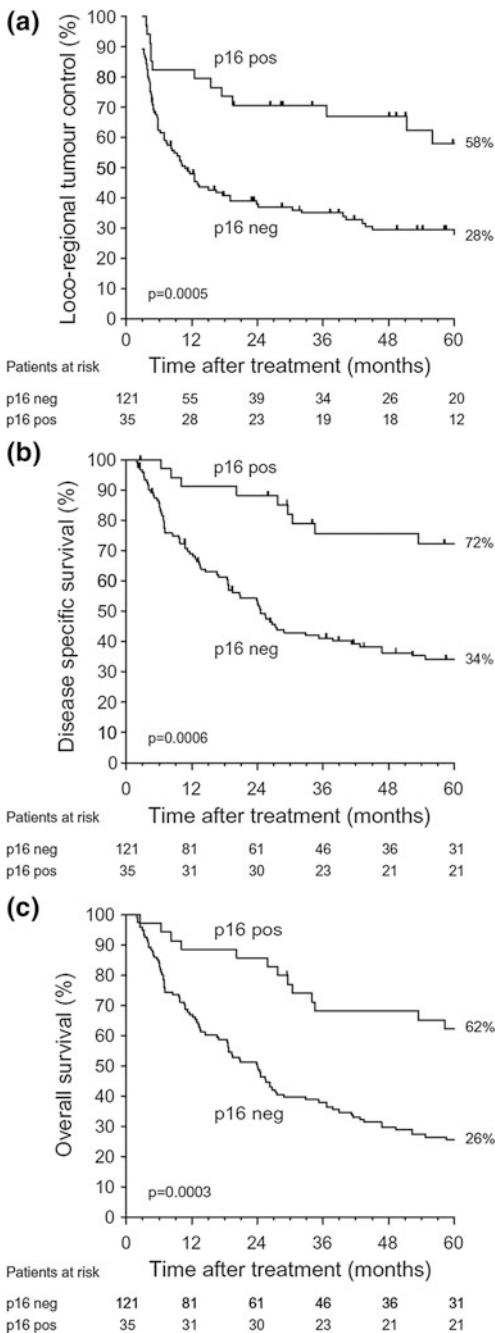
There are limitations to all methods of HPV detection, and which method to choose ultimately depends on the purpose of viral detection and consequently the extent of acceptable uncertainty of the test. Combined assays may represent an alternative way to reliably detect biologically relevant HPV infections in FFPE head and neck specimens, as proposed by Smeets et al. (2007). In previous randomized trials (Ang et al. 2010), p16-IHC was found to be a stronger prognostic factor for outcome than detection of HPV DNA by ISH and presently the most widespread clinical testing for tumour HPV status incorporates p16-IHC and ISH, either as stand-alone test or in combination (Jordan et al. 2012).

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## 6 Effect of HPV on Response to Single-Modality Radiotherapy of HNSCC

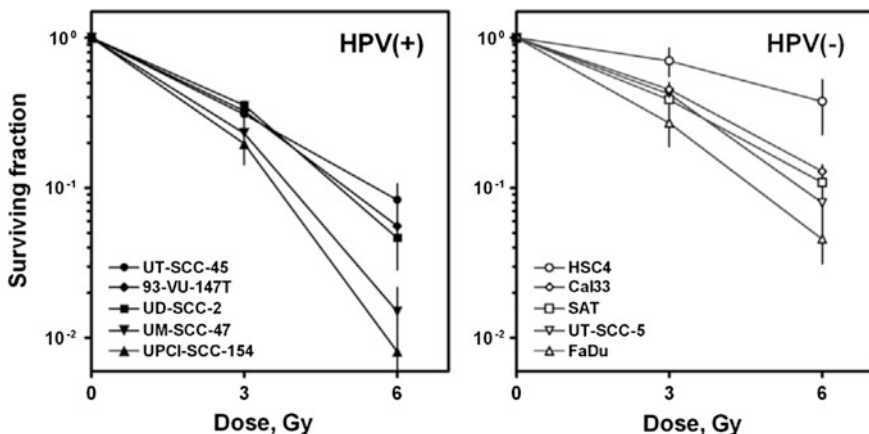
Although, at present, the use of conventional radiotherapy as single-modality treatment in locally advanced HNSCC has been replaced by altered fractionation and combined modality schedules, the influence of tumour HPV status on outcome has been investigated in a few original studies in which conventionally fractionated radiotherapy was the predominant treatment. Among the first to suggest HPV-positivity to be a favourable prognostic factor in HNSCC were Friesland et al. (2001), Mellin et al. (2005, 2000) and Lindel et al. (2001) based on their analyses of single-institution retrospective case series. Methodologically, the studies differed from each other in several aspects, including disparities between the endpoints, definitions of time to event and the reported estimates used to evaluate the prognostic impact of HPV, which complicates a direct comparison of the results. Nevertheless, although not unambiguous, their observations supported the hypothesis that HPV infection had a positive influence on radiotherapy outcome for patients with HNSCC. The studies were all included in a meta-analysis, predominantly based on retrospective case series (Ragin and Taioli 2007) which confirmed the favourable impact of HPV-positivity. The meta-analysis also demonstrated that apparently the beneficial prognosis was restricted to tumours of oropharyngeal origin. In a well-characterized and prospectively collected cohort of head and neck cancer patients treated homogeneously with conventional radiotherapy alone, evaluation of tumour HPV status, expressed by p16, demonstrated that p16 expression was the strongest independent determinant of loco-regional tumour control as well as disease-specific and overall survival (Fig. 3) (Lassen et al. 2009).

**Fig. 3** The influence of HPV-associated p16 expression on response to conventionally fractionated radiotherapy. Actuarial estimated loco-regional tumour control (a), disease-specific (b) and overall survival (c) rates in patients with p16-positive and p16-negative carcinomas of the pharynx and supraglottic larynx. With permission from Lassen P.



Thus, this study demonstrated a major impact of HPV-associated p16 expression on treatment response and survival in patients with head and neck cancers treated with conventionally fractionated radiotherapy alone.

The randomized DAHANCA 6 and 7 trials (Overgaard et al. 2003) compared the use of 5 fractions/week with 6 fractions/week, thereby shortening overall treatment time from 6½ weeks to 5½ weeks, while preserving the same total dose and fraction number. The study showed a significant benefit in favour of the 6 fractions/week schedule in terms of loco-regional control and disease-specific survival. Using p16 as retrospective stratification parameter within this prospective trial (Lassen et al. 2011) confirmed the strong independent prognostic impact of HPV-associated p16 expression on outcome after radiotherapy, both in terms of tumour control, disease-specific and overall survival. Moreover, the analysis suggested that HNSCC, regardless of tumour p16-status, reacts in a similar beneficial manner when treated with moderately accelerated fractionation compared to conventional fractionation as also shown in the updated meta-analysis of altered fractionation in HNSCC (Pignon et al. 2009). The cellular and molecular mechanisms underlying the favourable outcome for HPV-positive tumours treated with radiotherapy have recently been subject to further investigation. In preclinical investigation of HNSCC cell lines, Rieckmann et al. (Fig. 4) demonstrated a significantly higher radiosensitivity in HPV-positive cell lines compared to HPV-negative cells (Rieckmann et al. 2013). Furthermore, they found that, apparently, the increased radiosensitivity was due to accumulation of residual-DNA double-strand breaks (DSB) and associated with extensive G2 arrest in HPV-positive cells, indicating that the enhanced radio-responsiveness in HPV-positive tumours could reside in compromised DNA repair capacity of the cells.



**Fig. 4** Cellular radiosensitivity of HPV-positive and HPV-negative cell lines. Clonogenic survival of HPV-positive (*left*) and HPV-negative (*right*) cell lines after irradiation. With permission from Rieckmann T.

## 7 The Role of HPV in Hypoxic Modification During Radiotherapy

Hypoxia plays a pivotal element in radiotherapy of HNSCC and modification of hypoxia during radiotherapy improves loco-regional control, cancer-specific as well as overall survival (Overgaard 2011). In the DAHANCA 5 trial (Overgaard et al. 1998), patients were randomized to receive the hypoxic radiosensitizer nimorazole or placebo. A retrospective analysis of p16 status (Lassen et al. 2010) of 331 of the 414 patients in the original trial suggested that the response to hypoxic modification during radiotherapy differed by tumour p16 status. No significant benefit from nimorazole was observed in p16-positive tumours but only in the p16-negative tumours. In line with this are the observations from a retrospective analysis of another concept of hypoxic modification. In the TROG 02.02, “HeadSTART” (Rischin et al. 2010) trial from the Trans-Tasman Radiation Oncology Group, 853 patients with previously untreated locally advanced HNSCC of the oral cavity, pharynx, or larynx were randomized to conventional radiotherapy (70 Gy in seven weeks) concurrently with either high-dose cisplatin or cisplatin plus the hypoxia-selective cytotoxic drug tirapazamine. The original study failed its primary endpoint, but a later retrospective analysis in a small subset of these patients using HPV/p16 analysis suggested that only HPV-negative patients did benefit from treatment with tirapazamine (Rischin et al. 2010).

Toustrup et al. (2012) took another approach and tested a hypoxic gene expression classifier in the same patients from DAHANCA 5 as described above and found that nimorazole only improved loco-regional control in p16-negative, hypoxic tumours, although the hypoxic gene profile could also be found in a subset of the p16-positive tumours. The finding of hypoxic p16-positive tumours was confirmed, when estimating hypoxia using FAZA PET/CT imaging in HNSCC before curative radiotherapy (Mortensen et al. 2012) demonstrating that p16-positive and p16-negative tumours were equally hypoxic.

Sorensen et al. (2013) recently confirmed the pronounced radiosensitivity of HPV-positive HNSCC cells in a preclinical experiment. They also demonstrated that HPV-positive HNSCC cell lines display similar sensitivity under hypoxic conditions as the HPV-negative cell lines under normoxic conditions and moreover that under extreme hypoxia both HPV-positive and HPV-negative HNSCC cells benefit from hypoxic modification by nimorazole. Although, the experiment was performed under extreme hypoxic conditions, it is reasonable to suggest that the same results would be found under more moderate hypoxia. These preclinical findings suggest that HPV-positive tumour cells are hypoxic, but this hypoxia does not become of clinical significance because due to the extreme radiosensitivity of HPV-positive cells radiation doses of about 66 Gy will kill the cells anyhow, offsetting the effect of hypoxic modification by nimorazole.

## 8 The Role of HPV in Chemo–radiotherapy

HPV/p16 status has been estimated retrospectively in several studies involving chemo–radiotherapy (Attner et al. 2012; Fakhry and Gillison 2006; Lau et al. 2011). Ang and colleagues analysed the HPV/p16-status in 721 patients from the RTOG 0129 trial (Ang et al. 2010) randomizing between accelerated fractionation radiotherapy of 72 Gy/42 fractions in six weeks, with a concomitant boost of twice-daily irradiation for the last 12 treatment days versus 70 Gy/35 fractions over a seven-week period. Intravenous cisplatin was administered at a dose of 100 mg/m<sup>2</sup> of body surface on days one and 22 in the accelerated fractionation arm on days one, 22, and 43 in the standard fractionation group. The three-year rate of overall survival was similar in both arms, but when stratifying for HPV/p16 status, HPV-positive tumours did significantly better than the HPV-negative tumours using progression-free survival and overall survival as the endpoints. However, an analysis of whether the outcomes in the different chemo–radiotherapy arms differed by tumour HPV status is not available and an important purpose remains in exploring the optimal combination of radiotherapy and chemotherapy. The group from Princess Margaret Hospital in Toronto sought to elucidate this problem further in a non-randomized cohort of 505 oropharyngeal cancer patients treated with either chemo–radiotherapy (primarily high-dose cisplatin every third week) compared to primarily accelerated radiotherapy as single modality (O’Sullivan et al. 2013; O’Sullivan et al. 2012). They divided patients according to HPV status, stage and pack-years using the same algorithm as Ang and colleagues (Ang et al. 2010). Based on multivariate analyses using overall death and recurrence-free failure as endpoints as well as risk-profiling for having distant metastases, it seemed that HPV-positive oropharyngeal patients with T1-3 with N0-2b and less than 10 pack-years had minimal risk of distant recurrence irrespective of treatment with chemo–radiotherapy or radiation alone. Thus, these patients could be a possible target group for a prospective treatment de-intensification study. Nevertheless, one have to bear in mind that such studies are retrospective and conducted in an uncontrolled cohort consequently leading to a risk of introducing bias or confounders.

The use of a high-dose cisplatin schedule every third week or combination chemotherapy was the predominant treatment strategy in the above-mentioned studies. Recently, the first data from the registration protocol DAHANCA 18 were presented (Bentzen et al. 2015). In total, 227 patients with locally advanced HNSCC were treated with moderately accelerated radiotherapy, 66–68 Gy/33–34 fractions, six fractions per week and concomitant weekly low-dose cisplatin 40 mg/m<sup>2</sup>. Pretreatment tumour p16 status was determined upfront. For p16-positive tumours, the five-year loco-regional control and overall survival were 89 and 93 %, respectively. These data indicate that the use of this less toxic regime might be sufficient for HPV-positive locally advanced HNSCC instead of high-dose cisplatin regimes. The hypothesis is also supported by the results of a recent small pairwise-matched analysis of weekly low-dose platinum versus high-dose cisplatin,



showing no difference in survival between the two treatment schedules (Dobrosotskaya et al. 2014). However, since a non-inferiority randomized study of low-dose weekly cisplatin versus high-dose cisplatin every third week has never been (and probably never will be) conducted, no data exist to firmly address this question.

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## 9 The Role of HPV in Bioradiation

Expectations to bioradiation have been high since the first results of the randomized phase III trial of radiotherapy plus/minus cetuximab was presented in 2006 (Bonner et al. 2006) and updated in 2010 (Bonner et al. 2010). Based on the toxicity profile, it was suggested that bioradiation could be a reasonable alternative to concomitant chemotherapy in low-risk HPV-positive cancer. Supportive of this theory is the subgroup analysis performed along with the five-year update of the study, suggesting that the strongest benefit of additional EGFR-inhibition was found in younger patients with good performance and tumours with oropharyngeal origin, small T-stage, large nodal stage and consequently locally advanced disease (Bonner et al. 2010). This is exactly the characteristics of the patient with an HPV-positive tumour (Eriksen et al. 2010). One surprising piece of information from the five-year update is that it is primarily the tumours with a low expression of EGFR that seems to benefit from concomitant cetuximab. Moreover, low EGFR-expression seems to be a characteristic feature of HPV-positive tumours and might be explained by the deletion of E5 when the HPV genome is integrated in the host DNA during malignant progression (Burk et al. 2009). Tumour HPV/p16 status was never examined in the trial, but an influence of HPV on the results is likely. In a DAHANCA study cohort of 336 patients with oropharyngeal cancer, p16 and EGFR were examined according to previously described immunohistochemical methods (Lassen et al. 2009; Eriksen et al. 2004) and an inverse relation between the two markers was found: p16-positive tumours tended to express low levels of EGFR,  $p = 0.0001$ . This correlation was only present in the oropharynx (Lassen et al. 2013). Until now, no trial has upfront investigated the HPV/p16-status and balanced patients according to this in the treatment arms. In the DAHANCA 19 trial (clinicaltrials.gov NCT00496652) stratification according to site, stage and p16-status was done prior to randomization of patients to (chemotherapy) radiotherapy plus/minus the EGFR-inhibitor zalutumumab. The first data from this trial was presented recently and indicated that neither the p16-positive nor the p16-negative tumours did benefit from addition of zalutumumab to curatively intended (chemotherapy) radiotherapy. These findings were supported by retrospective data on 895 patients from the RTOG 0552 showing, that the effect of adding cetuximab to chemoradiation might result in competitive effects and the results presented at ASCO 2011 (Ang et al. 2011) indicated no benefit of adding an EGFR-inhibitor to chemoradiation. In contrast, in a recent study from Pajares et al. (Pajares et al. 2013), tumour blocks from 108 stage III/IV HNSCC patients treated

with chemo–radiotherapy or radiotherapy plus EGFR-inhibitors were retrospectively analysed in a non-randomized setting. They showed that in this limited series, only the HPV-positive tumours did benefit from concomitant cetuximab when overall survival was used as endpoint. The design allowed for bias and confounding and data should be considered with caution. The RTOG 1016 is the first prospective trial to randomize between chemo–radiotherapy with high-dose cisplatin and bio-radiotherapy with cetuximab in HPV-positive oropharyngeal HNSCC only. The study that was launched in summer 2011 is rapidly accruing and aims at 706 randomized patients.

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## 10 Perspectives for Future Treatment for HPV-Positive Tumours

In general, patients with HPV/p16-positive tumours have a very favourable prognosis and it is discussed how to reduce treatment burden without jeopardizing treatment outcome. To date, no prospective randomized data exist as guidance for treatment de-intensification of HPV/p16-positive HNSCC. Data suggest that loco-regional control and consequently disease-specific and overall survival are especially favourable in HPV/p16-positive non-smoking patients (Ang et al. 2010; O’Sullivan et al. 2013) and that they could be a candidate group for treatment de-intensification. However, as the risk of loco-regional recurrence becomes low, then the importance of distant recurrences might be of increasing importance (O’Sullivan et al. 2013) and HPV/p16-positive patients might even recur with an unusual metastasis pattern (Huang et al. 2010).

Based on the present evidence, data suggest that radiotherapy alone might be suitable for the low stages, HPV-positive HNSCC preferably with accelerated fractionation and presumably without hypoxic modification. The latter should ideally be estimated upfront by hypoxic profiling (Toustrup et al. 2012). For larger T-stages and higher nodal involvement (N2c/N3) chemo–radiotherapy still seems to be the choice, but the use of the less toxic weekly low-dose cisplatin schedule seems promising. Data on the replacement of chemotherapy with cetuximab is primarily driven by the idea that the latter is less toxic, but sufficient data do not exist at the moment. The RTOG 1016 study, mentioned above, will together with further exploration of the DAHANCA 19 trial hopefully provide the data needed. A third arm in the RTOG 1016 trial with accelerated fractionation radiotherapy only would have been optimal for answering these burning questions.

Other approaches are underway. Combination of radiotherapy with recombinant vaccines with inserted transgenes that code for E6 and E7 oncoproteins modified to remove their oncogenic potential and adjuvant human interleukin-2 (IL-2) are in progress and seems promising bearing the immunogenic potential of HPV in mind (Wansom et al. 2010). Testing of this vaccine in women with cervix HPV16 CIN grade 2/3 showed that seven out of eight patients were free of CIN 2/3 relapse and HPV 16 infection one year after treatment and therefore did not require conization (Brun et al. 2011). Another promising concept of immunotherapy would be

blockade of the programmed death 1 protein (PD-1), an inhibitory receptor expressed by T cells, that can overcome immune resistance (ideally, as performed by the HPV) (Topalian et al. 2012). The experience in HNSCC is still very limited, but the biological basis is promising (Badoual et al. 2013).

In summary, the present data support the hypothesis that de-intensification of treatment might be possible in selected groups of HPV-positive HNSCC. However, all data so far are based on retrospective studies and cautiousness should be taken, when interpreting the suggested risk stratifications, since these low-risk groups might not have the same result with de-intensified treatment. So far the radiation oncology community has only limited experience in de-intensification of treatment schedules and therefore further investigation should be done within clinical randomized controlled trials. Finally, focus in these years has been very much on the HPV-positive tumours with a good prognosis, but still a substantial part of the patients are HPV-negative, smoking patients with comorbidities and a rather poor outcome. Optimization of treatment in these patients is much warranted as well.

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## FDG and Beyond

Dirk De Ruyscher, Karin Haustermans and Daniela Thorwarth

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### Abstract

Although many PET tracers are in use, FDG still is the most widely used in clinical oncology practice. FDG therefore deserves an in-depth discussion, which is even more interesting because of the huge increase in the molecular biology of glucose metabolism. Obviously, other tracers are of increasing importance as well, and these will be discussed in short.

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### Keyword

Molecular imaging · Target volume · PET · FDG · Tracer · Hypoxia

Although many PET tracers are in use, FDG still is the most widely used in clinical oncology practice. FDG therefore deserves an in-depth discussion, which is even more interesting because of the huge increase in the molecular biology of glucose metabolism. Obviously, other tracers are of increasing importance as well, and these will be discussed in short.

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## 1 FDG

In order to understand thoroughly the biological meaning of FDG uptake, it is necessary to have some insight into the way glucose is taken up by cells and how this is regulated.

### 1.1 Uptake of Deoxyglucose

2-Deoxy-2-[<sup>18</sup>F]fluoro-D-glucose or FDG is still the working horse for clinical PET imaging in oncology. It exploits the preferential utilisation of aerobic glycolysis by cancer cells. Like regular D-glucose, FDG enters the cell via the glucose transporters GLUT1 and GLUT3, which are ATP-independent (Bensinger and Christofk 2012). FDG is, again like glucose, phosphorylated by hexokinase to FDG-6-phosphate. While glucose-6-phosphate is converted to fructose-6-phosphate by an isomerisation process and thus comes into the glycolysis pathway, or is oxidised to 6-phosphogluconolactone to come into the pentose phosphate pathway, FDG-6-phosphate is not metabolised. This is due to the lack of an oxygen atom at the C-2 position of the molecule. FDG-6-phosphate is unable to diffuse out of the cell, and the process of dephosphorylation is very slow. FDG-6-phosphate is thus trapped in the cell at a rate that is proportional to the glucose consumption. FDG uptake gives therefore information about the uptake of glucose by ATP-independent transporters, i.e. specific GLUTs, and hexokinase activity.

Many common cancers show a high FDG uptake, including lung, breast and colorectal carcinoma, but also melanoma and many lymphomas (Scheibler et al. 2012). However, some cancers are not uniformly FDG-avid on PET scans such as hepatocellular carcinoma and pancreatic and prostate cancer. Reasons for the low FDG uptake visualisation on PET scans include alternative energy sources for glucose such as fatty acids, poor perfusion of the tumour, low density of tumour cells or a high background signal, e.g. by bladder filling. Some malignancies such as well-differentiated hepatocellular carcinoma exhibit such high-level glucose-6-phosphatase that FDG-6-phosphate is, contrary to the situation in most cancer cells, dephosphorylated (Torizuka et al. 1995). FDG subsequently can leave the cell. Some cancer cells are dependent on ATP-dependent glucose uptake (Bettendorff et al. 2002). These receptors are not a substrate for deoxyglucose and are thus not visualised by FDG.

### 1.2 Transcriptional Regulation of Glucose Uptake

Glucose uptake is regulated via a complex and incompletely understood mechanism. The hypoxia-inducible factor 1 (HIF1) regulates the expression of glucose transporters and of most of the glycolytic enzymes (Semenza 2012). HIF1 upregulates glucose uptake and increases the expression of pyruvate dehydrogenase kinases (PDKs) that phosphorylate and inactivate the mitochondrial pyruvate

dehydrogenase complex. This enzyme controls the entry of pyruvate into the tri-carboxylic acid (TCA) cycle. In normal cells under normal oxygen concentrations, HIF1- $\alpha$  is constantly degraded, but in hypoxic conditions it is stabilised. Stabilised HIF1 increases the influx of glucose and stimulates glycolysis, but also decreases the pyruvate flux into the TCA cycle. Oxygen consumption is reduced and ATP production diminishes from 36 mol ATP per mol glucose to only 2 mol ATP per mol glucose in case of anaerobic glycolysis. However, HIF1 stabilisation occurs not only in hypoxia, but is also very common in many cancers in the presence of oxygen. It may be caused by enhanced transcription downstream of the PI3K/AKT/mTOR pathway. In this scenario of aerobic glycolysis (Warburg effect), 4 mol ATP is made per mol glucose. Both anaerobic glycolysis and aerobic glycolysis thus lead to increased FDG uptake.

The MYC oncogene upregulates many glucose transporters, glycolytic enzymes, PDK1 and lactate dehydrogenase A (Osthus et al. 2000; Shim et al. 1997; Dang et al. 2008). MYC thus enhances the Warburg effect by increasing glycolysis while at the same time decreasing the entry of pyruvate into the TCA cycle. At the same time, MYC activates the transcription of glutamine transporters and glutaminase-1, leading to a higher conversion of glutamine to glutamate (Gao et al. 2009), an important carbogen and nitrogen source, by repression of miRNA-23a/b expression (Gao et al. 2009).

The well-known tumour suppressor p53 suppresses glycolysis regulating fructose-2,6-bisphosphatase (Bensaad et al. 2006). At the same time, p53 promotes the oxidative phosphorylation by increased synthesis of cytochrome c oxidase-2 (Matoba et al. 2006). The loss of p53 expression in tumour cells will thus lead into increased glucose uptake and decreased oxidative phosphorylation.

In conclusion, FDG uptake is a reflection of the tightly regulated uptake of glucose and the Warburg effect. These complex mechanisms explain why FDG uptake is related to cell viability (Dooms et al. 2009), hypoxia (van Baardwijk et al. 2007) and cell density, but also to MYC amplification, p53 mutation, activation of PI3K/AKT/mTOR and many others. It therefore does not come as a surprise that FDG uptake is by no means specific for a given biological characteristic, but is related to many unfavourable features. FDG thus remains the most frequently used PET tracer in oncology.

### 1.3 Use of FDG in Radiation Oncology

As FDG has been shown to be taken up by the viable parts of most tumours, it is widely used for radiotherapy (RT) staging, therapy decision-making and treatment planning, but also treatment alteration, response monitoring and follow-up assessment (MacManus et al. 2009; Arens et al. 2011; Mak et al. 2011; Das and Ten Haken 2011; Bentzen and Grégoire 2011; Wahl et al. 2011; Nestle et al. 2009). Unfortunately, in some tumours FDG cannot be used in a reliable manner as its uptake is not visible due to FDG accumulation in surrounding normal tissues such as brain, stomach or heart or excreted FDG in the urine.

FDG PET has been shown to have a high impact on the diagnosis and staging of a variety of different cancers, such as lung, head and neck and colorectal cancer, melanoma, lymphoma, breast and gynaecological cancers as well as oesophageal tumours (Gambhir et al. 2001). Combined FDG PET/CT has been shown to perform superior-to-stand-alone RT planning CT in a number of different cancer sites such as lung, head and neck, or colorectal or gynaecological tumours (Delbeke and Martin 2004; Troost et al. 2010; De Ruyscher and Kirsch 2010; Lambrecht and Haustermans 2010; Haie-Meder et al. 2010).

In non-small cell lung cancer (NSCLC), FDG PET/CT is commonly used for staging, RT target volume definition and treatment planning. In prospective trials where FDG PET was used for tumour staging, about 20 % of the patients considered for curative RT were excluded from radical RT because of advanced disease diagnosed during FDG PET (Mac Manus and Hicks 2012). Incorporation of FDG PET into patient selection and RT treatment planning has led to significant improvements for patients with NSCLC (Mac Manus 2010). The use of FDG for RT planning allows better target volume definition, reduced inter-observer variability and selective irradiation of involved mediastinal lymph nodes (De Ruyscher et al. 2012; MacManus et al. 2009). Studies that compared PET-based target volume delineation to only CT-based contouring of the gross target volume (GTV) reported significant differences (Mac Manus and Hicks 2012). A recent clinical study reported on frequent changes in the management of patients with NSCLC due to FDG PET/CT findings and excellent association of FDG PET with overall survival (Mac Manus et al. 2013).

Also head and neck cancer (HNC) has been extensively studied with FDG PET/CT. Higher precision and better reproducibility have been observed when using FDG PET for the GTV definition (Grégoire et al. 2012; Arens et al. 2011; Mak et al. 2011; Gornik and Weber 2011; Nestle et al. 2009). Predictive value of FDG uptake before the start of RT has been reported recently (Picchio et al. 2013; Schinagl et al. 2011). Methodological approaches for the direct integration of PET information into RT planning are still under investigation (Picchio et al. 2013; Schinagl et al. 2011).

In the management of cervical cancer, FDG PET has been shown to be more sensitive than CT in detecting lymph node metastases (Grigsby 2009). Increased FDG uptake in para-aortic lymph nodes has been associated with lower control probabilities. Similarly, positive uptake in the primary tumour after the end of therapy was correlated with less favourable outcome (Grigsby 2009).

FDG is increasingly used for improved target volume delineation. A variety of different methodological approaches for manual, semi-automatic or automatic PET-based GTV contouring have been proposed and extensively studied in the last years (Shepherd et al. 2012; Erdi et al. 1997; Schaefer et al. 2008; Geets et al. 2007). In addition to large differences in complexity and implementation, validation of PET-based delineation strategies is scarce. Furthermore, different physical and biological factors as well as patient management and examination protocols may have a major impact on PET image characteristics such as spatial resolution, signal-to-noise ratio and tracer quantification. As a consequence, those factors will

also affect target volume delineation (Schinagl et al. 2013). Several studies that compared automated PET-based contouring to the GTVs delineated by trained radiation oncology specialists reported the superiority of manually delineated volumes (Schinagl et al. 2013; Bayne et al. 2010).

Data published so far on studies investigating the potential of FDG PET/CT in RT suggest two possibilities for the future use of FDG PET in RT: (i) PET image data are predictors but can only be used for patient selection or (ii) PET signal information can in addition be used to alter or intensify RT individually (Grégoire et al. 2012). The latter approach is called dose painting (DP) and aims at prescribing higher doses to volumes showing positive PET tracer uptake (Thorwarth et al. 2010). The idea of DP has been extensively studied in theoretical studies investigating the technical feasibility and the dosimetric effect of such treatment adaptations. Overall, two different DP strategies have been proposed so far: (i) homogeneously escalated dose level to a  $GTV_{PET}$  or (ii) prescription of locally varying increased doses according to the signal of each single PET voxel. To date, only a few studies were published that realised PET-based dose escalation in HNC or NSCLC (Madani et al. 2011; Berwouts et al. 2013; Van Elmpt et al. 2012).

In addition, a number of different studies have shown the value of FDG PET/CT for early response monitoring of (chemo-)radiotherapy in several tumour sites (Usmanij et al. 2013; Cuenca et al. 2013; Van Elmpt et al. 2012; Hatt et al. 2013). A recent study in oesophageal cancer investigated the potential of early response assessment via FDG PET/CT after 2 weeks of radiochemotherapy (20Gy plus 2 cycles of chemotherapy). The authors concluded that the metabolic response defined as  $1-SUV2/SUV1$  showed a great prognostic value in  $n = 72$  oesophageal cancer patients (Cuenca et al. 2013). Another recently published study reported similar results for a study aiming at response assessment with FDG PET/CT in  $n = 28$  NSCLC patients. In this study, early metabolic changes were measured using the difference in the total lesion glycolysis (TLG) between the pre-treatment FDG PET scan and the second scan at the end of the second week of therapy. Here, changes in TLG during the first two weeks of therapy were reported to be prognostic for response to concomitant radiochemotherapy in NSCLC (Usmanij et al. 2013). Furthermore, pre-treatment TLG was observed to be correlated with progression-free survival (PFS) in this patient group (Usmanij et al. 2013). A similar study published by Van Elmpt et al. (2012) investigated response assessment in  $n = 34$  NSCLC patients using changes in mean SUV in the target volume comparing pre-treatment FDG PET/CT scans to scans acquired after two weeks of radiochemotherapy. This study concludes that a decrease in FDG uptake in the primary tumour correlates with higher long-term overall survival, whereas changes in tumour volume defined on CT did not show a correlation with overall survival (Van Elmpt et al. 2012). Also for rectal cancer, the potential of early response prediction based on sequential FDG PET was shown in a clinical study (Hatt et al. 2013).

As a consequence, FDG PET/CT might in the future be a very efficient tool for early response assessment and eventually also for subsequent decisions on therapy alterations and adaptations.

In 2000, initially guidelines for the evaluation of response to treatment in solid tumours (RECIST) had been published (Therasse et al. 2000, 2006), which have been revised later (RECIST 1.1) (Eisenhauer et al. 2009). Those RECIST criteria were recommended to be used for the measurement and extrapolation of response to treatment in RT. Wahl et al. (Wahl et al. 2009) modified those criteria for dedicated use of FDG PET for response measurements and follow-up examinations (PET response criteria in solid tumours, PERCIST 1.0).

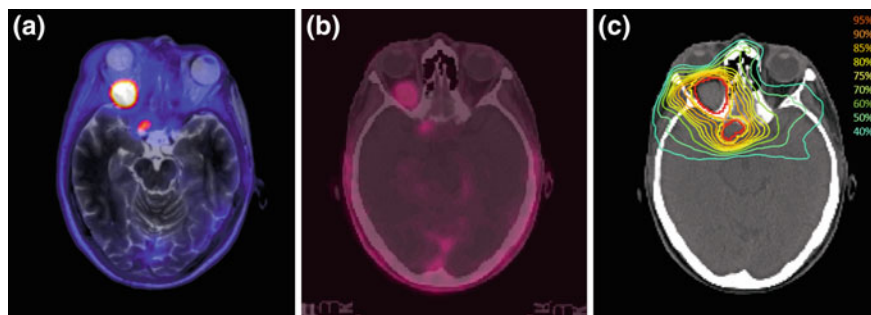
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## 2 Other Tracers, Excluding Hypoxia

The uptake of  $^{18}\text{F}$ -fluorothymidine ( $^{18}\text{F}$ -FLT), a marker of DNA synthesis, correlates with tumour proliferation (Apisarnthanarax et al. 2006; Buck et al. 2003; Muzi et al. 2005; Wagner et al. 2003; Yamamoto et al. 2007; Yap et al. 2006). FLT can detect changes in proliferation during and after irradiation in colorectal tumours and breast cancer cell lines (Pan et al. 2008; Roels et al. 2008; Wieder et al. 2007). FLT PET enables to image proliferation during chemo-radiotherapy (Everitt et al. 2009). FLT PET has recently been shown to allow for early prediction of therapy response in HNC (Hoeben et al. 2013).

Amino acid tracers may have the advantage over FDG in that they more specifically accumulate in viable cancer cells (Kubota et al. 1995). However, clinical data remain scarce (Grosu and Weber 2010). Amino acid PET is to date mostly used in brain tumours, as in the brain amino acids do not present with physiologic uptake as, for example, FDG does.  $^{11}\text{C}$ -Methionine (MET) PET is currently the most popular amino acid tracer used in PET imaging of brain tumours (Glaudemans et al. 2013). It provides a high detection rate and allows for accurate lesion delineation for RT treatment planning, especially in glioblastoma but also in other tumours such as metastatic brain tumours. A recent study showed that the usage of MET PET for the definition of target volumes planned for stereotactic irradiation with intensity-modulated RT (IMRT) may contain additional information compared to MRI, which remains the gold standard for stereotactic irradiations in the brain (Miwa et al. 2012). But also other amino acid PET tracers, such as O-(2- $^{18}\text{F}$ -fluoroethyl)-L-tyrosine (FET) PET may be very effective for RT treatment planning and also for the assessment of response to treatment. Galldiks et al. could show the added value of FET PET examinations in comparison with contrast-enhanced MRI data in glioblastoma (Galldiks et al. 2012). This study concluded that in contrast to volumes defined from gadolinium MRI, changes in FET PET may be a valuable parameter to measure treatment response in glioblastoma (Galldiks et al. 2012).

Choline PET/CT, labelled with either  $^{11}\text{C}$  or  $^{18}\text{F}$ , is mainly used for imaging of prostate cancer (Picchio et al. 2010). Nevertheless, for primary prostate cancer, the additional value of choline PET/CT to MRI has been shown to be very limited for the localisation of intraprostatic especially when multiparametric MRI is used (Van den Bergh et al. 2012, 2013). In contrast, for recurrent prostate cancer that has already been treated primarily with RT and present with suspicion for relapse



**Fig. 1** [68 Ga]-DOTATATE PET/MR imaging for accurate RT target volume delineation and treatment planning in meningioma. **a** Hybrid PET/MR imaging using [68 Ga]-DOTATATE. Small additional lesion close to the pituitary gland is only visible in the DOTATATE PET data. **b** Fusion of DOTATATE PET to the planning CT. **c** Planning CT with gross target volume (GTV) in red consisting of the two lesions visible in the PET/MR data in (a), planning target volume (PTV) in yellow defined as GTV plus a 2-mm expansion in all three directions. In addition, the clinical IMRT plan is shown represented by different isodose lines (see legend in the figure) highly conformal to the PET-based target volume. For treatment planning, the treatment planning system Hyperion (Eberhard-Karls University Tübingen, Germany) was used

during follow-up, the detection rate of choline PET is very high and has been reported to be proportional to trigger PSA (prostate-specific antigen) (Chondrogiannis et al. 2013). As a consequence, choline PET may have an important impact on the therapeutic strategy in patients with recurrent prostate cancer and can help to determine appropriate treatment (Soyka et al. 2012).

$^{68}\text{Ga}$ -DOTATOC or  $^{68}\text{Ga}$ -DOTATATE PET/CT is widely used for imaging of meningioma in order to accurately define target volumes for RT treatment (Thorwarth et al. 2011; Graf et al. 2013; Combs et al. 2013). Meningiomas express the somatostatin receptor subtype 2 (SSTR2) (Reubi et al. 1986); therefore, the  $^{86}\text{Ga}$ -labelled somatostatin receptor ligands DOTATOC and DOTATATE (Velikyan et al. 2012) can be used as tracers for the visualisation of the geometric extension of meningiomas with PET (Khan et al. 2009; Miederer et al. 2009; Henze et al. 2005). Several studies could show that DOTATOC PET/CT imaging is of additional value compared to MRI for RT target volume definition (Thorwarth et al. 2011; Graf et al. 2013; Combs et al. 2013) (Fig. 1).

### 3 Conclusions

FDG PET integration in RT is now standard practice and has improved patient care substantially. FDG will most likely remain the working horse in the near future in oncology, but new more specific tracers are emerging, some of which have been successfully introduced in daily clinical practice.

**Conflict of Interest** None to declare.

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# On the Reliability of Automatic Volume Delineation in Low-Contrast [ $^{18}\text{F}$ ]FMISO-PET Imaging

Robert Haase, Michael Andreeff and Nasreddin Abolmaali

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## Abstract

Hypoxia is a marker of poor prognosis in malignant tumors independent from the selected therapeutic method and the therapy should be intensified in such tumors. Hypoxia imaging with positron emission tomography (PET) is limited by low contrast to noise ratios with every available tracer. In radiation oncology appropriate delineation is required to allow therapy and intensification. While manual segmentation results are highly dependent from experience and observers condition (high inter- and intra observer variability), threshold- and gradient-based algorithms for automatic segmentation frequently fail in low contrast data sets. Likewise, calibration of these algorithms using phantoms is not useful. Complex computational models such as swarm intelligence-based algorithms are promising tools for optimized segmentation results and allow observer independent interpretation of multimodal and multidimensional imaging data.

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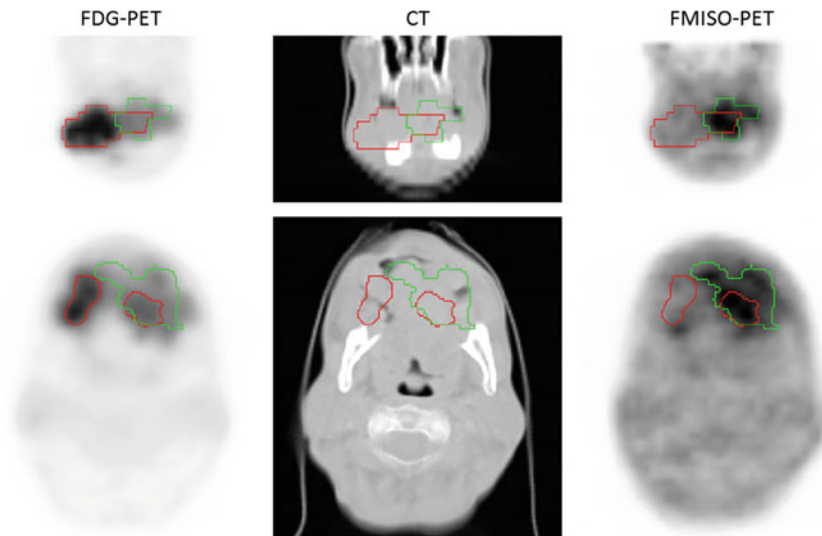
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### Keywords

Tumor microenvironment · Hypoxia imaging · FMISO-PET · Ant colony optimization algorithm · Swarm intelligence · Image analysis

## 1 Introduction

In diagnostic imaging for oncology, non-invasive detection and localization of biological properties in vivo like hypoxia of solid tumours is of increasing interest and based on several publications (Lee et al. 2008; Thorwarth and Alber 2010; Choi et al. 2010), numerous studies are ongoing to achieve a rational clinical application. Early work demonstrating the use of the tracer [ $^{18}\text{F}$ ]-fluoromisonidazole (FMISO) in positron emission tomography (PET) showed the capability of assessing hypoxia in vivo (Rasey et al. 1996). While FMISO-PET imaging remains promising (Le et al. 2010; Abolmaali et al. 2009), controversies about imaging accuracy and reproducibility are discussed (Nehmeh et al. 2008; Mortensen et al. 2010; Yasuda et al. 2013; Okamoto et al. 2013). Hypoxia imaging visualizes changes in the tumour microenvironment during therapy (Lee et al. 2009), and resulting imaging-derived parameters appear to be promising prognostic factors for the evaluation of therapy response (Rajendran et al. 2006; Eschmann et al. 2005; Hugonnet et al. 2011; Zips et al. 2012). On the other hand, it was also reported that



**Fig. 1** Using FMISO-PET, a new perspective on tumour biology could be added to the established imaging modalities. In this patient, a tumour at the base of the mouth contains two subvolumes comprising differences in biological properties: the right lateral part delineated in *red* accumulates FDG more than the part on the left side. The region of increased FMISO uptake outlined in *green* appears reverse

neither the presence nor the absence of increased FMISO uptake after four weeks of chemoradiotherapy may correlate with therapy outcome (Lee et al. 2009). Nevertheless, a major aim of hypoxia imaging is to inform the clinician on the presence of hypoxia and to support him in making therapeutic decisions. By adding a reliable method for hypoxia detection to the imaging procedure, the clinician could get another perspective to tumour biology as emphasized in Fig. 1. Tumour hypoxia may indicate decreased response independent from chosen therapy (Höckel et al. 1993) and individualizing therapy after analysis of biological imaging data may improve therapeutic success. Upcoming approaches in the field of radiotherapy, such as intensity-modulated radiotherapy (IMRT) and image-guided radiotherapy (IGRT), allow for the inclusion of biological aspects such as hypoxia into RT planning, but none of the proposed strategies (Thorwarth and Alber 2010; Lee et al. 2008; Madani et al. 2007) has reached wide acceptance in clinical practice yet. Other hypoxia tracers such as [ $^{18}\text{F}$ ]-EF5 and [ $^{18}\text{F}$ ]-FAZA have also been investigated (Komar et al. 2008; Beck et al. 2007; Souvatzoglou et al. 2007). But tracer uptake within the tumour is not higher using these tracers compared to FMISO (Souvatzoglou et al. 2007; Dubois et al. 2009; Grönroos and Minn 2007). Furthermore, image contrast is just at the same order of magnitude using FMISO. On the contrary, [ $^{18}\text{F}$ ]-fluorodeoxyglucose (FDG) PET delivers images containing significantly higher standard uptake values (SUV) within the tumour volume and lower SUVs in normal tissues. Accordingly, FDG PET images show an image contrast about one order of magnitude higher than when using FMISO (Abolmaali et al. 2011). Improvements of FMISO-PET image quality may be obtained by the following: 1) using new scanner technology such as improved detectors (Yasuda et al. 2013), 2) introduction of signal improving algorithms (Hofheinz et al. 2011), 3) developing imaging protocols producing data sets with the best achievable contrast (Abolmaali et al. 2011) and 4) methods for partial volume correction (Hofheinz et al. 2012). However, reliable target volume delineation is a prerequisite. Thus, it is worthwhile developing approaches for accurate delineation of hypoxic volumes in low-contrast FMISO-PET images. This article summarizes the abilities of standard threshold-based segmentation approaches in this field and the given limitations. Alternatives utilizing more complex algorithms are outlined.

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## 2 Application of Conventional Threshold-Based Algorithms to FMISO-PET

In FMISO-PET image analysis, standardized thresholds were already applied to differentiate between normoxic and hypoxic tissues (Rajendran et al. 2006; Swanson et al. 2009). The possibility of rough differentiation between these tissues cannot be denied, since hypoxic tissues actually may show higher uptake than surrounding tissues. But the resulting delineation should be treated with caution. On the one hand, the methodology of applying thresholds to volume data is of limited accuracy because of the given spatial resolution of clinical PET scanners. A PET scanner may not be able to measure hypoxia-induced high-tracer uptake if necrosis and hypoxia are mixed inside the tumour volume (Swanson et al. 2009) or even worse a small amount of

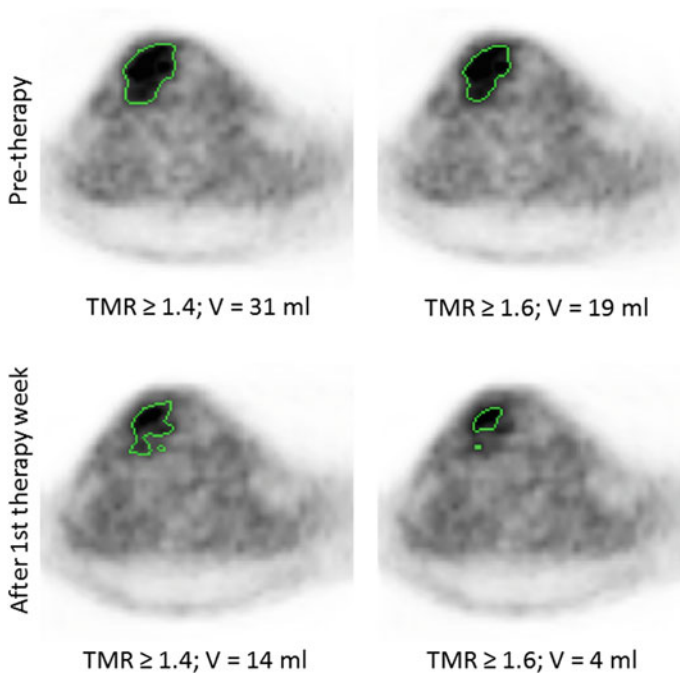
hypoxic cancerous stem cells survive in a necrotic environment. Pronounced perfusion in adjacent regions may have a similar effect on FMISO uptake in hypoxic regions (Cho et al. 2009). Currently, there is no reference standard for hypoxia quantification in vivo beside invasive pO<sub>2</sub>-measurements (Chapman et al. 2001) and the development of a non-invasive reference quantification is not foreseeable. Furthermore, correlation between FMISO-PET and pO<sub>2</sub>-measurements using Eppendorf electrodes was not observed in a clinical set-up, but FMISO-PET delivered lower hypoxia measurements compared to Eppendorf pO<sub>2</sub> measurements (Mortensen et al. 2010). Therefore, the expected high false-negative rate of FMISO-PET in assessing hypoxia is difficult to measure in a clinical environment, where Eppendorf electrodes are not part of the examination routine.

Considering FMISO-PET images show higher uptake within hypoxic tumour volumes poses the question as to whether such a volume can be delineated correctly using a single predefined threshold. This aspect has been investigated for FDG PET imaging of peripheral lung lesions (Biehl et al. 2006), where the contrast between FDG avid lesions and normal lung parenchyma with very limited FDG uptake is high. The authors showed that even in this preferable situation, no single threshold applied to PET data is capable to delineate volumes accurately compared to reference delineations from computed tomography (CT). This conclusion is expected to be transferable to FMISO-PET since the same tomography technique is used and comparable biological mechanisms are generating the uptake. Nevertheless, results are expected to be even worse since FMISO-PET reveals significantly lower contrast between normal and hypoxic tissues. However, hypoxia measurements resulting from FMISO-PET imaging may allow predicting local progression free survival based on thresholding even though image quality is limited (Zips et al. 2012).

In general, there are three straight-forward ways for determining the threshold to segment PET data:

- The first is to assume a relative threshold depending on the mean activity measured in blood samples (Swanson et al. 2009; Thorwarth et al. 2005).
- Secondly, the threshold can be determined relative to the maximum activity inside tumour volume (Nehmeh et al. 2009).
- Thirdly, a threshold level can be applied that depends on the mean activity measured in a reference region of the PET data set (Eschmann et al. 2005; Abolmaali et al. 2011).

When determining an appropriate threshold and verifying it by manual visual observation, it depends not only on surrounding tissue, the pertinent organ and technological aspects, such as the chosen reconstruction algorithm. The chosen threshold and resulting final delineation depends also on the individual investigator and applied display window settings (Nestle et al. 2005). A similar effect of image characteristics to delineations was observed for automatic segmentation algorithms (Cheebsumon and van Velden 2011; Cheebsumon and Yaqub 2011). Furthermore, small changes on the threshold may in one case result in contour changes which are hard to see but in other cases may lead to dramatic volume differences. This fact, highlighted in Fig. 2, may be



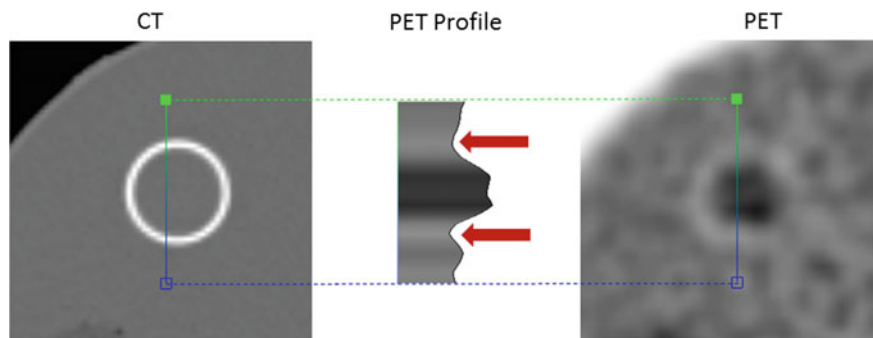
**Fig. 2** Different target-to-muscle-ratio (TMR) thresholds applied on two FMISO-PET data sets acquired before therapy and after the first therapy week. The choice of the threshold appears decisive for volume delineation. Using a threshold of  $TMR \geq 1.4$  leads to the conclusion that the hypoxic volume halved during one week, the threshold  $TMR \geq 1.6$  suggests volume reduction by quartering. The observed differences are a result of the applied threshold, not entirely a result of biological properties

of importance when applying constant thresholds to repeated FMISO-PET imaging data to observe therapy progress. Varying thresholds may alternatively change the measured reproducibility of FMISO-PET. The work of Nehmeh et al. (2008) is often cited to underline limited reproducibility of FMISO-PET itself, but the authors found a strong correlation of activity distribution in patients scanned twice using FMISO-PET. The correlation decreased dramatically after applying a fixed background-dependent threshold to the data sets, as proposed by Rajendran and analysing the remaining voxels (Rajendran et al. 2006). One source of this aspect of limited reproducibility in FMISO-PET may be the threshold approach and not solely the imaging technique. Additionally the technique of using voxel-wise Pearson correlation coefficient seems to be inadequate to measure reproducibility of serial PET scans (Schwartz et al. 2011; Westgard 2008). The observations of Nehmeh et al. that FMISO-PET is of limited reproducibility was furthermore contradicted in a recent study applying similar analysis methods to a different patient cohort (Okamoto et al. 2013).

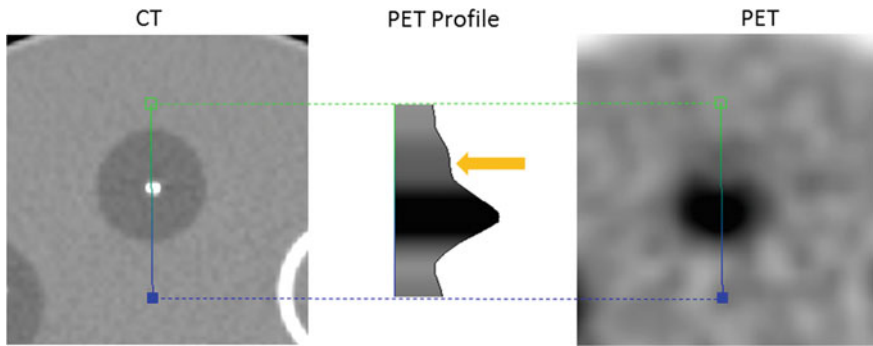


### 3 Limitations of Phantom Experiments for Calibration of Threshold-Based Algorithms

A widely accepted approach to determine an appropriate threshold for volume definition in PET imaging is the use of phantom experiments for calibrating segmentation algorithms (Nehmeh et al. 2009; Schaefer et al. 2008). Several groups utilized cylinder phantoms containing glass spheres for such measurements. Cylinder and spheres contained a radionuclide, but the mean activity concentration inside and outside the spheres differed. Varying activity levels were set up by using the same radionuclide but different solutions in spheres and cylinder to achieve constant activity ratios. Alternatively two different radionuclides in spheres and cylinder were placed. The radionuclides have different half-life, and thus, mean activity concentrations within sphere and cylinder will decrease differently with time. Resulting images show varying contrast with time (Haase et al. 2010). On the one hand, gathering images, when using the same radionuclide, is time-consuming requiring several phantom experiments. Using different nuclides is more efficient but introduces differing mean positron ranges in the imaging process. At the boundaries of the target objects, a gradient may appear in the dual radionuclide set-up, which cannot be generated by a single radionuclide. But this effect is expected to be negligible, since the limited spatial resolution of clinical PET scanners may not allow for measuring it. Using spheres with glass walls introduces another source of error in the process. There is no activity inside the glass walls, and therefore, the boundary between sphere and cylinder can be delineated more accurately than in patient data sets having no glass sphere around the target volume. This effect was surveyed earlier (Hofheinz et al. 2010; van den Hoff and Hofheinz 2013), and its importance for low-contrast PET was emphasized: thresholds determined in phantom experiments may not be appropriate if background activity is high in respect to the mean activity concentration in a target volume. Additionally the PET-detectable walls of the spheres, shown in Fig. 3, can lead to artefacts in the attenuation map used for correction of the PET signal. This fact leads to the idea of using wax spheres without



**Fig. 3** In the CT data set of a phantom experiment, the glass walls of a target simulating sphere are clearly visible. However, if the ratio between target and background activity is low enough, in this case 1.5, the sphere also becomes visible in the corresponding PET image and the profile through the target volume (*red arrows*)

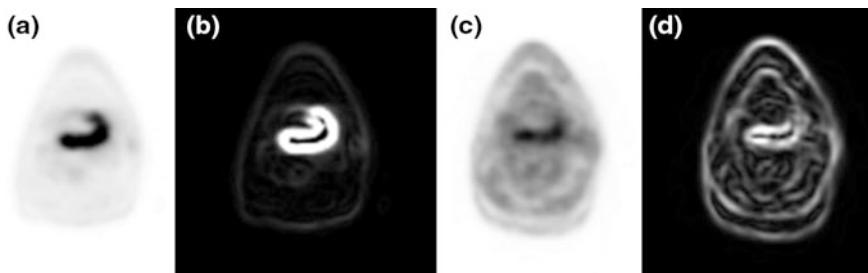


**Fig. 4** When utilizing wax spheres as target volume simulation in a phantom experiment, the target volume may appear homogeneous in CT. But in the corresponding PET image, it is obvious that a subvolume of the sphere shows decreased signal intensity. The region is depicted in the profile through the target sphere with an *orange arrow*

inactive walls (Bazañez-Borgert et al. 2008). But creating wax spheres with homogeneously distributed activity concentration is technically challenging (Haase et al. 2010). Resulting PET imaging data may show cold areas within the hot spheres as shown in Fig. 4. However, this kind of phantom experiments performed on a combined PET/CT scanner in principal offers the opportunity to calibrate and validate segmentation algorithms in general because of the CT-measurable volume of the spheres as reference measurement. But for a final evaluation of a determined threshold more sophisticated algorithms, the new methods should be validated by applying them to clinical data sets.

#### 4 Image Contrast Limits Abilities of Established PET Segmentation Algorithms

Our investigations encouraged us to accept the hypothesis that there is no threshold-based algorithm which generates appropriate volume delineations for FMISO-PET. Major challenges of PET image segmentation, such as inhomogeneities and non-spherical target volumes, were reviewed before (Lee 2010). In addition, FMISO-PET segmentation is complicated by a low ratio of activity, i.e. the low contrast, between target volume and surrounding tissue. Threshold-based algorithms allow FDG PET data sets to be segmented more accurately. The edge between target and background is high due to high activity ratio between the tissues. But it must be stated that even when analysing FDG PET, estimation of the optimal threshold remains challenging. In non-small cell lung cancer (NSCLC), where the activity concentration in the surrounding lung tissue is low, the boundary of the target object can be segmented using a range of algorithms (Schaefer et al. 2008; Nehmeh et al. 2009; Hofheinz et al. 2012; Jentzen et al. 2007). But when analysing FMISO-PET data sets of the head and neck region, we found that the mean target-to-muscle-ratio (TMR) is often less than 2:1 (Abolmaali et al. 2011). The values of an amount of voxels inside the presumptive hypoxic subvolume may be equal or lower than grey



**Fig. 5** Edge detection filter (Sobel operator) was applied to axial **a** FDG PET and **c** FMISO-PET images of a patient with hypopharyngeal cancer. Resulting gradient images are shown in **b**, **d**. The pixels in the filtered images **b** and **d** are white, if a high activity gradient is present in the surrounding pixels, and black, if the gradient is negligible. In the FMISO-PET image, several edges are visible and the edge of the target volume is not clearly differentiable

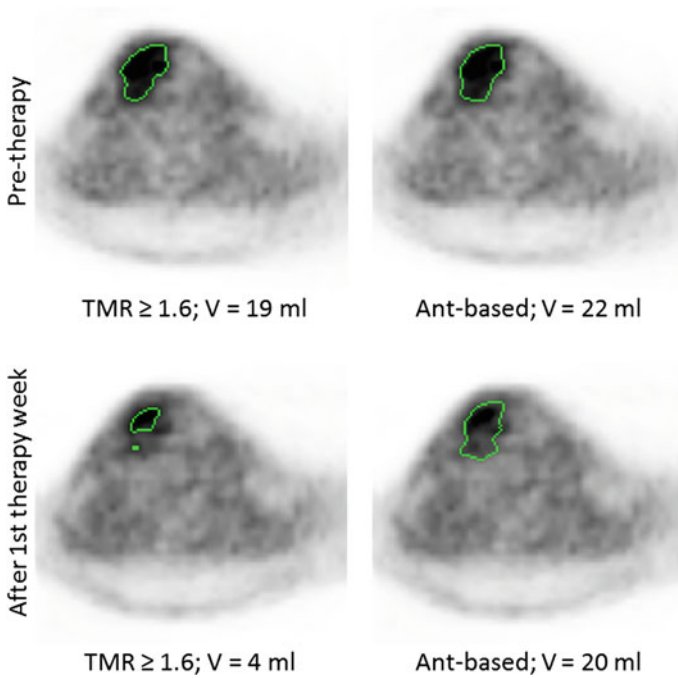
values of the surrounding tissue. The contrast is insufficient to differentiate tissue based directly on voxel values. This finding seems to be independent on the method; neither histogram-based algorithms nor methods calibrated by phantom measurements may deliver accurate delineations.

Gradient-based methods may also not be suitable to overcome these issues, because without high ratios between the structures to differentiate, there is no steep gradient which can be utilized as boundary between the volumes. To visualize the effect on gradient-based methods, two gradient images of an FDG and an FMISO-PET data set are shown in Fig. 5. It demonstrates that gradients between target and background in FMISO-PET are not distinguishable from gradients within or outside the target volume. Signal fluctuations between different tissues may be misinterpreted as boundary of a false-positive target volume.

## 5 Further Developments on Segmentation Algorithms for Low-Contrast PET Data

Nevertheless, there are several approaches published that may be able to deliver improved segmentation results when applied to low-contrast PET data such as FMISO-PET. Stated key aspects used multimodal information (Yu et al. 2009), spatial information (Montgomery et al. 2007) and intelligent algorithms (Haase et al. 2011; Hsu et al. 2008; Sharif et al. 2010). Methods like these may allow overcoming issues such as volume reproducibility in repeated FMISO-PET, explained in the first paragraph, as shown in Fig. 6.

All of these methods have still to be validated in detail for low-contrast FMISO-PET patient data. They are also connected by the fact that additional information to the value of voxels is utilized to find the solution to the segmentation problem. The measured uptake inside a single voxel alone does not allow its classification. This single activity value needs to be connected with data from other imaging modalities and/or with



**Fig. 6** When applying the ant-based segmentation algorithm proposed by our group (Haase et al. 2010), increased contour reproducibility and volume stability can be observed in repeated FMISO-PET compared to standard thresholds like  $TMR \geq 1.6$

voxels in its neighbourhood. In particular, in head and neck cancer, several different tissues are surrounding the periphery of the tumour compared to the central parts. Therefore, an approach is needed which is able to differentiate between the volumes having different properties in each imaging modality. For example, determining intersections between FMISO-PET indicated hypoxia and limited perfusion shown on dynamic contrast enhanced (DCE) magnetic resonance imaging (MRI) may deliver reliable information for interpreting the tumour microenvironment correctly (Cho et al. 2009). The combined analysis of FDG PET and CT data, currently becoming standard in clinical routine, offers advantages to tumour delineation (Nestle et al. 2009), and the number of automatic delineation approaches using the combination of FDG PET and CT is increasing. This trend may be the first step in introducing fully automatic, observer independent, multimodal segmentation approaches utilizing more than just two imaging modalities. First investigations in the field of multiparametric analysis of tumour microenvironment using different PET tracers, CT and MRI are promising (Kawai et al. 2011; Dirix et al. 2009). The research focus on FDG PET/CT fusion in the recent years ensures this approach to be a safe basis for combining several views of the same tumour and to deliver more perspectives than each modality could contribute alone.

## 6 Conclusions

Threshold-based algorithms for volume segmentation in PET were not found to be applicable for segmenting low-contrast data accurately. These algorithms may be able to show trends like decreasing hypoxic subvolume during therapy. But a single threshold for accurate delineation in one patient data set is not expected to deliver precise results in another data set, even of the same patient. If tracer uptake and thus image quality cannot be further improved, it is worthwhile to develop new algorithms for image processing. A new technique for accurate delineation of hypoxic subvolumes is urgently needed, especially for low-contrast data sets like FMISO-PET. To increase delineation accuracy, not only improved imaging techniques and protocols are needed, but also more intelligent, multimodal and complex segmentation algorithms must be developed. The trend of utilizing multimodality information in therapy planning is promising due to the dependencies between different biological aspects, which can be measured using modern imaging techniques. But the complexity of multimodal and multidimensional information interpretation may exceed human abilities, because comparing more than two imaging modalities with each three or four dimensions in image space is not a trivial task. Therefore, simple understandable algorithms should be combined with complex computational models, e.g. swarm intelligence-based algorithms, allowing for better interpretation of multimodal and multidimensional information.

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# FMISO as a Biomarker for Clinical Radiation Oncology

Sebastian Zschaeck, Jörg Steinbach and Esther G.C. Troost

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## Abstract

Tumour hypoxia is a well-known negative prognostic marker in almost all solid tumours. [18F]Fluoromisonidazole (FMISO)-positron emission tomography (PET) is a non-invasive method to detect tumour hypoxia. Compared to other methods of hypoxia assessment it possesses some considerable advantages: It is non-invasive, it delivers spatial information on the hypoxia distribution within the entire tumour volume, and it can be repeated during the course of radio (chemo)therapy. This chapter briefly describes different methods of hypoxia

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evaluation and focuses on hypoxia PET imaging, with the most commonly used tracer being FMISO. The preclinical rationale and clinical studies to use FMISO-PET for patient stratification in radiation therapy are discussed as well as possible agents or radiation-dose modifications to overcome hypoxia.

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**Keywords**

Hypoxia · FMISO · [18F]Fluoromisonidazole · PET · Positron emission tomography

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## 1 Background on Tumour Cell Hypoxia

Tumour heterogeneity plays a pivotal role in several solid tumours regarding the outcome after surgery, radiotherapy and chemotherapy. Amongst the various tumour characteristics, tumour cell hypoxia is the most relevant in the field of radiation oncology. Hypoxia is generally differentiated into chronic, i.e. diffusion-limited hypoxia caused by rapid tumour growth with insufficient neo-vascularization and impaired oxygen supply, and acute hypoxia, i.e. acute decrease in perfusion due to functional impairment of the (neo-) vasculature. Tumour cell hypoxia is known to negatively affect patients' outcome irrespective of the chosen therapeutic approach (surgery, radiotherapy) as shown in a landmark study for cancer of the uterine cervix (Hockel et al. 1996). It is known to promote local tumour growth, lymph node involvement and distant metastases formation. In radiotherapy, the unfavourable therapeutic effect may be caused by hampering the formation of free radicals after photon irradiation, a prerequisite for the envisaged biological effect. This phenomenon can be expressed by the oxygen enhancement ratio (OER) that compares the biological efficacy of radiotherapy under oxic versus anoxic conditions.

Several strategies to improve outcome in patients presenting with hypoxic tumours have been developed and used in clinical studies. These include the following: hyperbaric oxygen breathing, carbogen breathing or the additional application of hypoxia sensitizers to the standard treatment (Jordan and Sonveaux 2012; Janssens et al. 2012). Furthermore, the nitroimidazole derivative nimorazole has been extensively studied in several Danish studies and is now routinely used for Danish head and neck squamous cell carcinoma (HNSCC) patients. Cytotoxic agents activated under hypoxic conditions include tirapazamine (Rischin et al. 2010) or the novel agent TH-302 (Borad et al. 2014). The benefit of hypoxia modification (by various means) in HNSCC even without stratification for the hypoxic status has been shown by a large meta-analysis by Overgaard, analysing 4805 patients treated within 32 randomized clinical trials (Overgaard 2011). With modern radiation techniques facilitating highly selective dose delivery, the concept of the biological target volume, introduced by Ling et al. (Ling et al. 2000) in 2000, has gained interest. For hypoxic tumours are radiation resistant, an increase in

radiation dose, either to the whole tumour volume or selectively to the hypoxic subvolumes of the tumour only (dose painting), is aimed for. The latter approach can be either performed on a (manually or semi-manually) delineated subvolume or by prescribing individual doses to every voxel (dose painting by numbers) (Thorwarth et al. 2007). Obviously, it is important to assess the level of hypoxia both globally and on tumour subvolume level before and during (chemo)radiotherapy in order to facilitate these dose-painting techniques.

This chapter briefly addresses invasive measures to quantify hypoxia, but focuses on positron emission tomography (PET) of hypoxia-related markers as they may repeatedly image the entire tumour volume, and even more importantly, this technique is non-invasive.

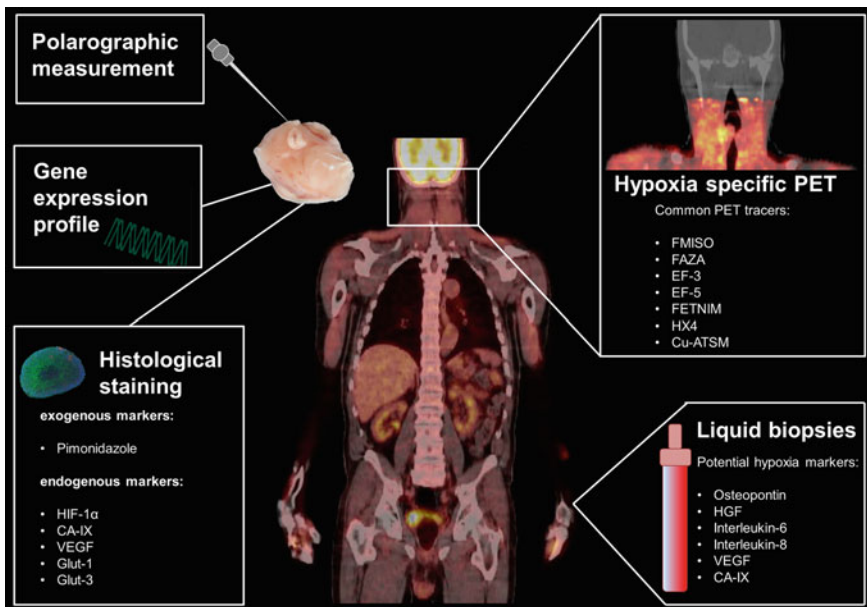
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## 2 Invasive Assessment of Tumour Cell Hypoxia

The classical gold standard for determination of the oxygen partial pressure has been the Eppendorf electrode. For its easy accessibility, the longest experience for pre-therapeutic Eppendorf electrode measurements exists in carcinomas of the uterine cervix (Hockel et al. 1996; Hoogsteen et al. 2009; Yaromina et al. 2006). This technique holds some important drawbacks: it has an invasive nature, is labour-intensive and cannot be used in all solid tumours for poor accessibility. Furthermore, Eppendorf electrode measurements only analyse small portions of the tumour and are unable to distinguish between vital hypoxic and anoxic necrotic tumour subvolumes.

Another attempt to measure tumour cell hypoxia is the immunohistochemical staining of histological samples that were obtained from the tumour or lymph node metastases. There are both exogenous markers, i.e. they need to be administered to the patient via an infusion or tablet, and endogenous markers, i.e. upregulated hypoxia-related markers. The most commonly used exogenous marker currently considered the reference standard is pimonidazole. Pimonidazole detects hypoxia below 10 mmHg (1.3 kPa) and has been used in preclinical and clinical studies (Hoogsteen et al. 2009; Yaromina et al. 2006). In the past, it required intravenous administration approximately 30–60 min before gathering the biopsy, but meanwhile an oral form exists and is FDA-approved. For logistic and financial reasons, endogenous markers of hypoxia have been pursued. These include hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ), carbonic anhydrase-IX (CA-IX), vascular endothelial growth factor (VEGF) and the glucose transporters 1 and 3 (Glut-1 and Glut-3) (Troost et al. 2005; Ogawa et al. 2011; Goethals et al. 2006; Bussink et al. 2003). Noteworthy, the expression of these endogenous markers is influenced by a plethora of mechanisms and not exclusively driven by hypoxia. In accordance with Eppendorf electrode measurement, immunohistochemical staining of biopsies only represents a fraction of the entire tumour, is burdensome for the patients and is not ideal for repeated measurement of hypoxia.

Another attempt to quantify hypoxia is the use of serological blood biomarkers. Osteopontin is probably the best-established blood marker in the context of radiotherapy. In a HNSCC tumour cell line, hypoxia was found to upregulate osteopontin via a Ras-activated enhancer (Zhu et al. 2005). Plasma osteopontin correlated inversely with pO (Jordan and Sonveaux 2012) measured by Eppendorf electrodes in both HNSCC and NSCLC patient cohorts, and with worse clinical outcome in a variety of solid tumours (Le et al. 2003, 2006; Buijssen et al. 2014; Ostheimer et al. 2014). However, the factors influencing osteopontin levels and the additional value of combining several blood biomarkers (Osteopontin, VEGF and CA-IX) are subject of ongoing research (Ostheimer et al. 2014; Lukacova et al. 2005). A fourth strategy assessing tumour hypoxia is genetic analysis. Toustrup et al. (Toustrup et al. 2011) identified a hypoxia gene expression profile in vitro and validated the classifier in vivo in HNSCC xenograft models. In the latter model, they additionally checked whether tumour heterogeneity affected the gene profile, i.e. whether biopsies from mixed hypoxic and oxic areas yielded representative results compared to autoradiography with a hypoxia-related PET tracer. They found measurable upregulation of hypoxia genes even in tumour biopsies taken from mixed hypoxic and oxic subvolumes as identified with autoradiography PET imaging. Figure 1 gives an overview of established hypoxia detection methods.



**Fig. 1** Overview of different hypoxia detection methods

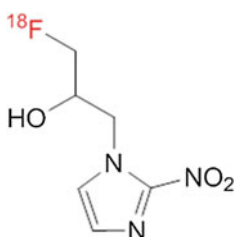
### 3 PET Imaging of Hypoxia and Relevance in (Radiation) Oncology

A non-invasive method for the detection and quantification of tumour cell hypoxia is PET imaging with hypoxia specific radiotracers, amongst which imidazole derivatives are most commonly used. Imaging hypoxia via PET has several advantages for radiotherapy compared to the aforementioned methods: The entire tumour volume can be assessed at several time points prior to and during treatment, and it can be used for dose-redistribution based on biological tumour characteristics, e.g. hypoxia (Hendrickson et al. 2011). Notably, [ $^{18}\text{F}$ ]Fluoro-deoxyglucose (FDG) may inherit some information on intratumoural hypoxia due to upregulation of Glut-1 by HIF-1 $\alpha$ , but is aspecific and should, therefore, not be used for hypoxia imaging (Van Baardwijk et al. 2007).

Already in 1979, J.C. Chapman from the University of Alberta, Canada, recognized the importance of hypoxic sensitizers for radiation therapy and shortly thereafter he utilized  $^{14}\text{C}$ -labelled misonidazole for the imaging of hypoxic areas in murine tumours (Chapman et al. 1981; Chapman 1979). He described the importance as future markers for hypoxic cells in tumours with potential clinical applicability (Garrecht and Chapman 1983). For diagnosis of ischaemia, Mathias et al. (1987) presented a first concept for radiotracers to be applied in nuclear medicine techniques based on hypoxic sensitizers.

Since then, [ $^{18}\text{F}$ ]Fluoromisonidazole (FMISO) has become the hypoxia PET tracer most commonly used in a variety of solid tumours (Rajendran and Krohn 2015). Figure 2 shows the chemical structure of FMISO.

Additional imidazole tracers, including 5-[ $^{18}\text{F}$ ]fluoro-5-deoxy-D-arabinofuranosyl-2-nitroimidazole (FAZA), [ $^{18}\text{F}$ ]nitroimidazole-*N*-trifluoropropyl-acetamide (EF-3), [ $^{18}\text{F}$ ]nitroimidazole-*N*-pentafluoropropyl-acetamide (EF-5), [ $^{18}\text{F}$ ]fluoroerythronitroimidazole (FETNIM), or [ $^{18}\text{F}$ ]fluoro-nitro-*H*-imidazol-methyl-*H*-triazol-propanol (HX4), have mainly been designed to improve the slow accumulation in comparison with FMISO within the tumour, and to enhance the signal-to-background ratio (SBR). Beside them, the SPECT or PET radiotracer 5-[ $^{123/124}\text{I}$ ]iodo-5-deoxy-D-arabinofuranosyl-2-nitroimidazole (IAZA) is available (Reischl et al. 2007). Non-Imidazole tracers, e.g. [ $^{64}\text{Cu}$ ]Cu-diacetyl-bis-*N*-methylthiosemicarbazone (Cu-ATSM) are less frequently used and may also reflect



**Fig. 2** Chemical structure of FMISO, the most important PET hypoxia marker

tumour perfusion rather than being a hypoxia-selective tracer (Movahedi et al. 2012). Due to the poor availability of  $^{64}\text{Cu}$  a broad application is not visible. All these hypoxia specific tracers, except for Cu-ATSM, share the necessity of a relatively long interval between tracer injection and imaging and therefore are merely a surrogate of chronic instead of acute hypoxia. Off note, there are various additional imaging methods for the assessment of hypoxia, including blood oxygenation level-dependent (BOLD) magnetic resonance imaging (MRI) or optical spectroscopy, but discussion of these is beyond the scope of this chapter (Horsman et al. 2012; Chitneni et al. 2011).

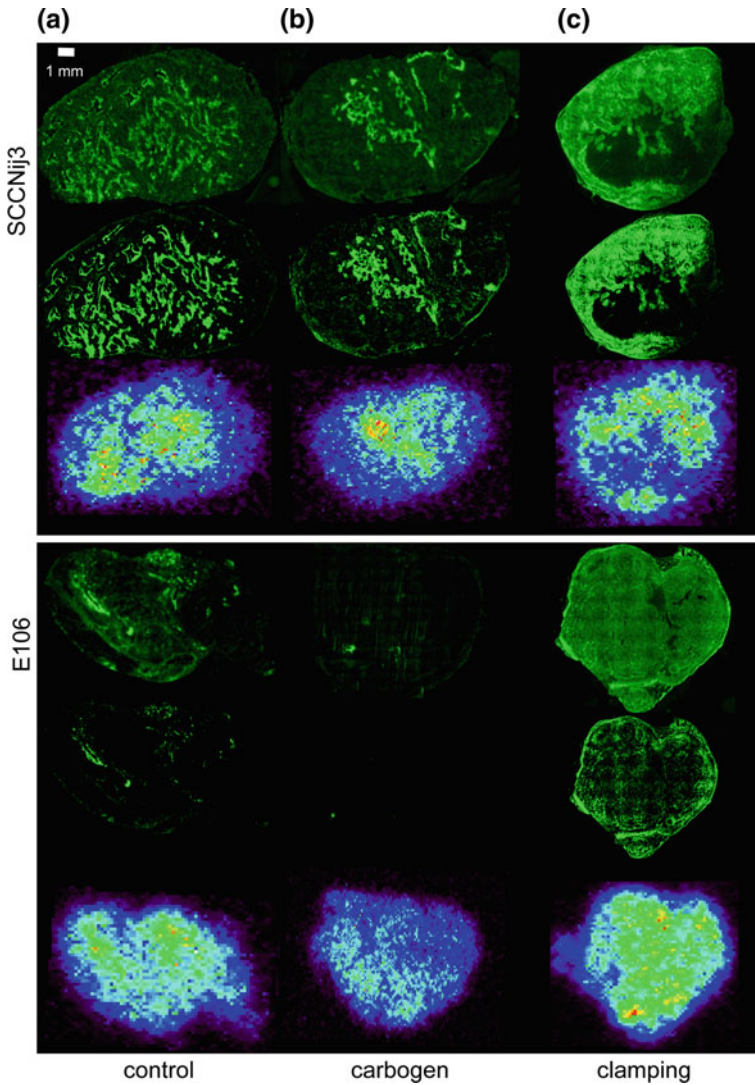
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## 4 Preclinical Validation Studies

Many *in vivo* studies focused on the correlation of different methods for hypoxia detection in order to move from invasive to non-invasive ones. One study compared FMISO uptake in rodent tumour xenograft models with robotic-guided multiple  $\text{pO}_2$  measurement via electrodes and found a high non-concordance in some individual data pairs, possibly explained by partially necrotic, i.e. anoxic, subvolumes that do not take up the tracer (Chang et al. 2009). Numerous studies have shown a reasonably good correlation between hypoxic subvolumes as detected by pimonidazole immunohistochemistry and FMISO and FAZA uptake by autoradiography and/or microPET, under varying levels of oxygenation or artificially induced clamp hypoxia (Troost et al. 2008; Busk et al. 2008, 2009, 2013; Troost et al. 2006). Figure 3 depicts examples of pimonidazole-stained and FMISO-autoradiographed xenografts under different oxygen conditions.

In a study comparing the aforementioned hypoxia tracers FMISO, FAZA, HX4 and Cu-ATSM with pimonidazole and CA-IX immunohistochemistry in a head and neck xenograft tumour line, all except for Cu-ATSM showed similar distributions within the tumour (Carlin et al. 2014).

Comparing the different tracers *per se*, modelling as well as preclinical studies have suggested a superior SBR of FAZA compared to FMISO, however, data are conflicting (Busk et al. 2013; Busk et al. 2009). Peeters et al. (2015) recently compared FMISO, FAZA and HX4 in rats bearing syngeneic rhabdomyosarcoma R1 tumours. As expected and probably due to its high lipophilicity, the maximum SBR for FMISO was not reached until 6 h, as compared to 2 h for FAZA and 3 h for HX4. Remarkably, whereas all three tracers were able to monitor artificially induced hypoxia, only FMISO was able to successfully depict hypoxia-modifying treatment with nicotinamide and carbogen. Finally, the spatial reproducibility of FMISO in two consecutive scans obtained within a 48-h interval was best. The low conformity of FAZA shown in this study is somehow surprising for another study showed a good reproducibility of FAZA hypoxic volumes, even after fractionated radiotherapy (Busk et al. 2013). In other publications, FMISO and HX4 were both able to detect decreased levels of hypoxia after carbogen breathing (Troost et al. 2006; Dubois et al. 2011), and FAZA uptake decreased after pure oxygen breathing (Piert et al. 2005).



**Fig. 3** Pseudo-coloured grey-value pimonidazole images (*top row*), images after segmentation of the pimonidazole signal (*middle row*), and FMISO autoradiography images (*bottom row*) of head and neck squamous cell carcinoma (SCCNij3) and glioblastoma (E106) xenograft tumour lines for control tumours, after carbogen breathing and clamping. Figure taken with kind permission from (Troost et al. 2006)

Data on Cu-ATSM are conflicting. Cu-ATSM has neither shown a good correlation with pimonidazole and CA-IX staining in solid tumour models, nor with FAZA uptake (Yuan et al. 2006; McCall et al. 2012). A correlation with FMISO uptake and oxygen probe measurement existed when applying an exceptional long time period between tracer injection and acquisition (O'Donoghue et al. 2005).



When investigating the value of hypoxia imaging, pre-therapeutic FAZA-PET was found to predict the therapeutic efficacy of adding nimorazole to radiotherapy in a preclinical sarcoma, but not in a glioma model (Bol et al. 2015). In rhabdomyosarcoma and NSCLC tumour models, Peeters et al. (2015) showed an association between the HX4-PET-derived hypoxic volume and tumour growth delay after treatment, and a benefit of the novel hypoxia specific cytotoxic agent TH-302 when combined with single-dose radiotherapy. In a recent study in HNSCC xenografts, Schütze et al. (2014) asserted the role of the FMISO-derived hypoxic volume and possible implications for subsequent dose escalation. Tumours with hypoxic volumes below the median had a significantly better local control rate after single-dose radiotherapy than those with hypoxic volumes above median. Interestingly, an increase of the single dose by another 10 Gy lead to similar increases of local control for both subgroups. Hence, tumour hypoxia as depicted by FMISO-PET is probably not barely a shift on the dose-effect curve for tumour control probability. If this had been the case, the same dose increase would have led to a much lower gain in tumour control in the hypoxic tumour subgroup. Rather, other effects most probably also play a role. The observations by Schütze et al. justify an attempt to moderately escalate the dose in hypoxic tumours to substantially increase the tumour control probability.

Taken together the preclinical data support the strong role of preclinical FMISO-PET-imaging as it correlates well with other established methods of hypoxia detection, it has a high reproducibility, and it reflects relevant therapy-induced changes.

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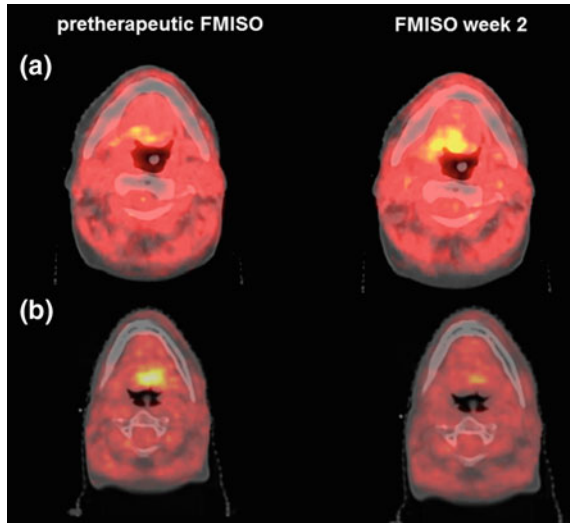
## 5 Clinical Studies

After primary or (neo)adjuvant (chemo)radiotherapy, hypoxia in particular affects treatment outcome in tumours with unsatisfactory local control rates, e.g. glioblastomas, locally advanced NSCLC and locally advanced HNSCC (Mortensen et al. 2012; Rischin et al. 2006). In a recent review article, the prognostic value of PET-measured hypoxia, in particular FMISO-PET, for local tumour control has been highlighted (Rajendran and Krohn 2015). Moreover, a large meta-analysis underlined the positive effect of hypoxia modification on local tumour control and treatment outcome (Overgaard 2007). In order to select patients benefitting from treatment modification and to prevent those patients not benefitting from suffering undesired treatment-related side effects, methods to stratify patients are mandatory.

Stratification may be based on relatively simple parameters including tumour necrosis, on plasma markers, or a hypoxia gene set (Eustace et al. 2013). However, for aforementioned reasons, non-invasive imaging is of particular interest. Several recent studies have shown the prognostic value of hypoxia PET imaging regarding outcome after (chemo)radiotherapy in various solid tumours (Mortensen et al. 2012; Servagi-Vernat et al. 2014; Zips et al. 2012; Bollineni et al. 2014; Zegers et al. 2013). Zips et al. (2012) highlighted the prognostic value of FMISO-PET imaging obtained after one and two weeks of (chemo)radiotherapy in a cohort of 25 HNSCC patients.



**Fig. 4** Example of 2 patients treated with cT3N2cM0 oropharyngeal cancer and FMISO-PET imaging. Patient **a** showed residual hypoxia and presented local recurrence 5 months after treatment. Patient **b** showed dissolution of hypoxia; this patient remained recurrence-free during follow-up



Conversely, FMISO-PET imaging obtained before treatment did not correlate with outcome. Figure 4 shows two patients of this study with sequential FMISO scans. In a small study cohort, Dirix et al. (2009) investigated FDG- and FMISO-PET, T1- and T2-weighted MRI, diffusion-weighted-(DWI-)MRI and dynamic contrast-enhanced (DCE-)MRI at various time points before and during primary radiotherapy in 15 HNSCC patients. Apart from the prognostic value of DWI- and DCE-MRI for locoregional recurrence, the prognostic value of FMISO-PET was underlined. A Danish study also found FAZA-PET imaging to stratify patients into groups according to loco-regional control (Mortensen et al. 2012). FMISO-PET imaging was part of a phase II clinical study on hypoxia-modification in patients with advanced HNSCC. In a substudy on 45 patients, FMISO-PET imaging prior to chemoradiotherapy was found to be a predictive marker selecting patients benefitting from the addition of tirapazamine (Rischin et al. 2006). In the cohort treated with chemoradiotherapy, 8 of 13 patients with tumour cell hypoxia as depicted by FMISO-PET experienced local relapse, whereas in the experimental arm, only 1 of 19 patients with hypoxia developed local recurrence.

Although older publications have shown conflicting results, recent studies have reported a good spatial reproducibility of repeated FMISO-PET-based hypoxic subvolumes obtained prior to initiation of (chemo)radiotherapy (Okamoto et al. 2013). Regarding reproducibility during treatment, Bittner et al. (2013) analysed the size, location and overlap of FMISO-PET positive subvolumes in 16 HNSCC patients. In patients with persistent hypoxia after 2 weeks of chemoradiotherapy, the FMISO-positive subvolumes mostly remained geographically stable. Summarizing these data, they prompt the scientific community to integrate hypoxia PET imaging into radiation treatment planning for hypoxia-directed dose escalation strategies.

At present, a German mono-institutional randomized phase II clinical trial investigates the clinical feasibility of FMISO-PET-based dose escalation in HNSCC (Welz et al. 2014). Patients in the standard arm undergo a conventional total dose of 70 Gy (chemo)radiotherapy, whereas in the experimental arm, patients receive 10 % dose escalation, i.e. 77 Gy, to the FMISO-PET-defined hypoxic subvolume. The planned interim analysis for the first 20 patients recruited into this study showed that dose escalation in this order of magnitude is well tolerated. Additionally, results of this study have thus far confirmed a prognostic model relating the dynamic FMISO-PET data to the individual tumour control probability established in an earlier study (Thorwarth et al. 2014).

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