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Diagnostic Optical Imaging of Breast Cancer: From Animal Models to First-in-Men Studies

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1. Introduction

The use of light-driven imaging techniques for the diagnosis of breast cancer dates back to as early as 1929. Transillumination was evaluated for potential differentiation of breast lesions, e.g. to distinguish benign cysts from solid tumours. Cutler summed the technique to be "...a simple procedure and a valuable aid in the interpretation of pathological conditions in the mammary gland. Its use is recommended in the routine examination of the breast..."(Cutler, 1929, 1931).

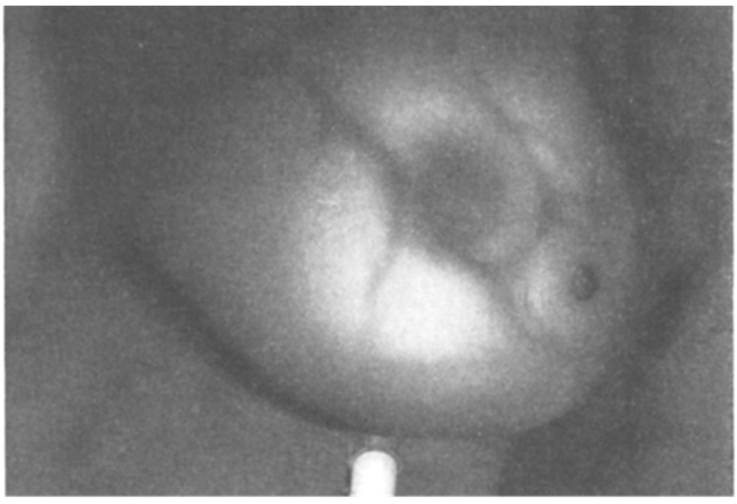

Fig. 1. Opacity on transillumination of a solid tumour in the breast (Cutler, 1931, reproduced with permission from Wolters & Kluwer).

At the same time, x-ray mammography emerged, routinely used for detection and diagnosis of breast cancer from the 1950s (Leborgne, 1951). Only relatively low sensitivity and specificity of mammography findings in combination with significant x-ray exposure though always triggered the parallel search for alternative diagnostic approaches. Over the following years, the use of near infrared (NIR) light was discussed to further facilitate

transillumination of breast tissue (Watmough, 1982a; Watmough, 1982b). Its unique characteristics regarding interaction with tissue - low absorption and reduced scattering – enable NIR light to travel through tissue and thus to reach deep tissue sections, therefore defining a valuable tool in diagnostic optical imaging (OI) techniques (Weissleder & Ntziachristos, 2003). Originating from transillumination, diffuse optical tomography (DOT) was initially thought to be capable of identifying tumour formation in anatomical regions accessible to NIR light, as tumour tissue is characterized by distinct optical properties, based on altered tissue architecture, blood flow and oxygen consumption. However, the limitations of this technique, especially the limited spatial resolution, have been illustrated in several publications. Although the advances on the field of instrumentation were significant, first clinical studies in breast cancer patients proved discriminating lesions regarding their malignant potential reflected by their absorption characteristics still to be challenging (Boas, 1997). To further improve the specificity of OI, fluorescence contrast agents, fluorophores operating in the NIR range of the spectrum, have been used. Three different types of optical probes are currently available for clinical or preclinical (basic research) purposes – unspecific, specific and so-called smart probes. The only clinically approved fluorochrome, indocyanine green (ICG) is an unspecific, perfusion-type contrast agent. First promising results of ICG-driven DOT for detection and differentiation of breast lesions have recently been published, confirming sensitivity and specificity (Ntziachristos et al., 2000). So far restricted to preclinical use only, specifically binding optical contrast agents, targeted on key-structures of cancer cells, allow for disease specific imaging and *in vivo* characterization of lesions down to the molecular level. As state of the art anatomical imaging frequently fails in detecting early effects of modern anti-tumour therapy, contrast agents targeted to different tumour cell epitopes have been described and used to sensitively detect the molecular characteristics of malignant lesions and their alteration under therapy (Achilefu, 2004; Achilefu et al., 2000; Becker et al., 2001). So called smart probes even increase sensitivity and specificity of OI. Fluorescence characteristics of these probes change upon interaction with specific target structures, e.g. proteinases, therefore allowing detection of vital, invasive tumour lesions with unrivalled sensitivity (Bremer et al., 2001c; Bremer et al., 2002). This review gives an overview about the approaches, recently made on the field of optical tracer development and evaluation with a focus on targets relevant for breast cancer diagnosis and characterization. Especially the visualization of angiogenesis as a key process in tumour progression is emphasized. Many factors that are involved in angiogenic events have been evaluated over the last decades and are now well understood. Several growth factors, their receptors and diverse pathways triggering their expression, are crucially involved in cancer progression and metastasis. Moreover some proteinases, especially matrix metalloproteinases (MMPs) and cathepsins, proved to contribute to tumour growth by e.g. degradation of extracellular matrix (ECM) components. Clinical translation of the efforts made in the past recent years will be the main challenge for researchers active on the molecular imaging field.

2. Instrumentation

Technically, optical imaging is based on the detection of light and the visualization of photon distribution in tissue. While visible light (380-700 nm) is almost completely absorbed, scattered and reflected in the uppermost sections of tissue already, light of the NIR range of the spectrum can be facilitated for *in vivo* imaging approaches. Light of around

650 – 950 nm is absorbed to a much lesser extent than light of lower wavelengths and can thus be detected in deeper tissue sections (Ntziachristos et al., 2003). Moreover, autofluorescence of tissue is minimal in this so called optical window (Fig. 2), resulting in a higher signal-to-background ratio for near infrared images (Bremer et al., 2001b).

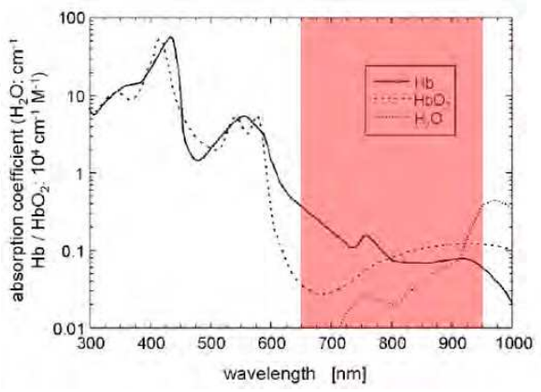

Fig. 2. The optical window between 650 and 950 nm, where absorption by water haemoglobin and deoxyhaemoglobin is minimal.

For non-enhanced optical imaging approaches, simple opposed lightsource detector pairs are used. Light is applied to the scan volume at a distinct place and the detector is used to collect the non-absorbed photons. Highly absorbing lesions (e.g. tumour lesions) are supposed to delineate as negative contrast. Diffuse optical tomography (DOT) is based on the same principle, creating 3D absorption maps from e.g. the female breast (Ntziachristos et al., 2000; O'Leary et al., 1995). Commercially available optical imaging devices use adapted light for dye excitation; in experimental settings ultrasound was alternatively used successfully for the excitation of fluorophores (Razansky et al., 2008; Yuan & Liu, 2010). Fluorescence-producing proteins, encoded by so-called imaging target genes can either be light-excitable or metabolism-dependent, producing fluorescence upon interaction with exogenous substrates (Chudakov et al., 2010; Lukyanov et al., 2010). All dyes regularly used for targeted *in vivo* imaging require excitation with light of distinct wavelength to produce a specific emission. An imaging unit therefore always consists of an excitation light source on the one hand and a signal recording camera on the other (Fig. 3). The animal is placed either between both for optical tomography (Fluorescence Mediated Tomography – FMT) or on the ground of an imaging chamber with light source and camera both on one side (Fluorescence Reflectance Imaging – FRI) (Mahmood et al., 1999; Weissleder et al., 1999). Reflectance imaging approaches in this context basically resemble traditional photography, allowing for acquisition of 2D image data at video rate with highly sensitive CCD cameras. Regularly, cameras are adapted with filter sets for each particular wavelength, virtually blocking diffuse background fluorescence. The image acquisition is preceded by illumination of the whole field of view with either light of distinctive wavelengths – adapted for the applied dye – or unfiltered, multi-wavelength light (Graves et al., 2004). The latter of course results in excitation not only of the applied dye but of virtually all substances capable of producing fluorescence. This circumstance contributes to the background signal and thus requires more sophisticated filter equipment at the signal recording side. Further increase of signal-specificity can be achieved by using excitation light of discrete wavelengths, using

e.g. filters or even more precisely laser-light. The main disadvantage of such laser-light equipped units is the reduced flexibility, as upgrading the system for detection of a new spectrum of dyes with different spectral characteristics would always require a new laser-light source, which is usually much more expensive than a new set of filters for multi-wavelength excitation devices. State of the art FRI systems frequently allow for fusion of fluorescence images with white-light and x-ray images.

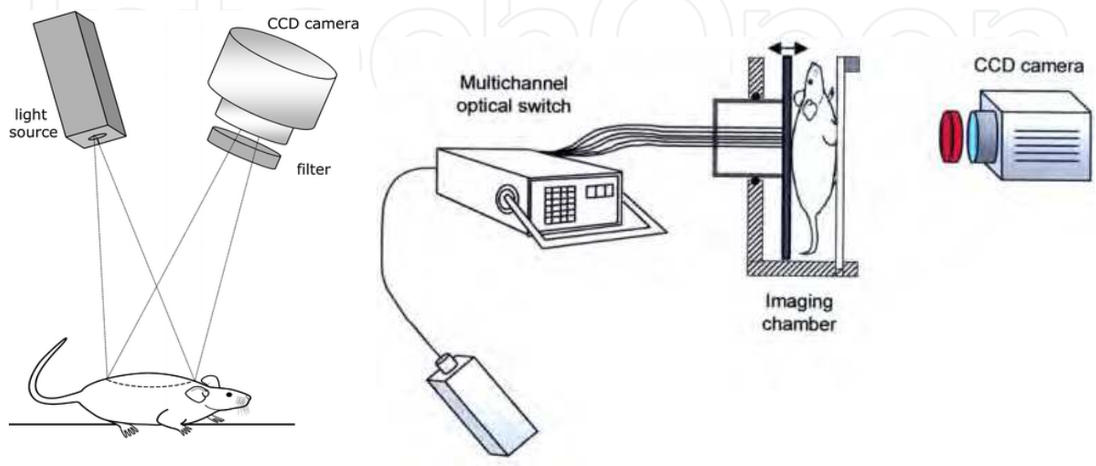

Fig. 3. Left: Typical FRI system for *in vivo* animal investigations. The illumination source and CCD camera are on the same side of the animal. Right: Example of an FMT scanner. The scanner uses multiple light sources and detectors to effectively collect light at multiple projections around the animal's body (from Graves et al., 2004; Ntziachristos et al., 2003; reproduced with permission).

Anatomical correlation of FRI results, though, is limited due to the basic imaging principle. Absorption and scattering of light in tissue hamper detection and correct visualization especially of such signals originating from deeper regions of the scan volume which may thus not be properly reflected by the signals obtained at the scan volume surface (Montet et al., 2005). Multi-angle imaging is frequently regarded as one possible solution for this general FRI limitation. First approaches on this field are promising regarding the propriety of signal-to-source correlation, but are still in a prototype stage. Moreover, multi-angle optical imaging does still not offer anatomical co-information and thus lacks the possibility to correct fluorescence signals for scattering and absorption yet. In first studies, combination of optical tomography and x-ray based computed tomography (CT) is used to overcome this limitation. CT is used to acquire anatomical information and thus to depict optical properties of tissue for more precise prediction of the origin of detected photons (Guo X. et al., 2010b; Nahrendorf et al., 2009). Commercially available FMT systems aim to solve this problem without additional anatomical data acquired by CT or MRI. A 2D scan matrix is excited point by point with dye-adapted laser-light while the resulting fluorescence signals are recorded on the other side of the animal. In a first step, the resulting fluorescence signals are used to calculate a scattering map of the scan volume. In a second step, the specific dye-associated signals are recorded and a 3D dataset is calculated on the basis of the scattering map and presumptions about the optical properties of the examined tissue (Stasic et al., 2003). The mathematical algorithm underlying this reconstruction of a 3D dataset from a

matrix of 2D data is based on the Born approximation to solve the wave equation (Vinegoni et al., 2009). The approach has been validated, using surgical implants of known concentrations of fluorescent dyes implanted into animals at defined points (Ntziachristos et al., 2002). The presumptions on optical properties of the heterogeneous tissue on the track of a single photon, though, can only be approximations and may cause inaccurate results in individual cases. Combination with established cross-sectional imaging for the creation of fusion images is on the way and may help to correct for those adverse results. Additionally, anatomical co-information will simplify interpretation of fluorescence signals, especially in complex models of disease.

The more modern translation into patient diagnosis started with the development of the first clinical optical breast imaging systems. Companies and academia put a lot of effort in this task, and in addition to several prototype instruments, three systems are commercially available at the moment. The Computed Tomography Laser Mammography system CTLM®, developed by Imaging Diagnostic Systems Inc., is a fully tomographic system and generates volumetric images of the breast (http://www.imds.com/ctlm/). Poellinger (Poellinger et al., 2008) and Floery (Floery et al., 2005) have used the system for small study groups and concluded that CTLM could be used for the delineation of malignant tissue but should be seen as an adjunct to conventional mammography only. The ComfortScan® system, distributed by Danum International Ltd., is a transillumination system that requires breast compression to generate 2D-images (http://www.danum.com/comfortscan.html). Fournier and colleagues concluded that the system had the potential to distinguish benign from malignant lesions but were not certain about specificity of findings and finally assumed a higher number of false positive results compared to conventional mammography (Fournier et al., 2009). The SoftScan® system by Advanced Research Technologies Inc., is a system that requires slight breast compression but is able to generate tomographic images of the breast (http://www.art.ca/en/clinical/). Using this system, van de Ven performed a phantom study, testing contrast agents of different intensity. It was concluded that the use of such contrast agents, at best in addition to a targeting ligand, would have a great potential in future optical breast cancer diagnosis (van de Ven et al., 2011). Detectors for fluorescence signals can also be minimized and e.g. integrated in endoscopic devices. Jaffer in this context reported the use of intravascular NIRF imaging in murine models of atherosclerosis (Jaffer et al., 2008; Jaffer et al., 2009).

3. Optical imaging of tissue perfusion

Due to the fact that oxy- and deoxyhaemoglobin are mainly responsible for absorption of NIR light in tissue, non-enhanced OI, e.g. transillumination, has been explored for detection of breast cancer, reflected by elevated tissue perfusion (Hawrysz & Sevick-Muraca, 2000). In 1931 already, Cutler described that naive transillumination diaphanography could help to identify breast cancer (Cutler, 1931). Decades later, DOT allowed for 3-D determination of scattering and absorption properties of tissue or turbid material, respectively. Both approaches nevertheless fail to provide sufficient physiological information for safe delineation of malignant lesions. And so, low specificity of imaging findings and reduced sensitivity for lesions in deeper tissue regions always promoted efforts to develop optical contrast agents. Proof-of-concept DOT mammography experiments employing ICG as a contrast-enhancing agent successfully showed that breast lesions could be delineated from healthy tissue, although the poor water-solubility and the resulting rapid liver-uptake

hampers the use of ICG in routine breast cancer imaging (Ntziachristos et al., 2000; Riefke et al., 1997). Other carbocyanine-based dyes with a more hydrophilic character were designed and employed in imaging experiments, as well. Especially SIDAG (1,1'-bis-(4-sulfobutyl)indotricarbo-cyanine-5,5'-dicarboxylic acid diglucamide monosodium; Global Drug Discovery, Bayer Schering Pharma AG, Berlin, Germany) shows improved photophysiological and pharmacological characteristics. This dye was developed by Licha and co-workers in the mid-nineties (Licha et al., 1996, 2000; Riefke et al., 1997) and showed good target-to-background contrast in a number of different murine xenograft models (Fig. 4), including breast cancer. A correlation of SIDAG-signal intensity to the vascular volume fraction of the tumour as an MRI-based parameter of angiogenic potential could be shown (Wall et al., 2008).

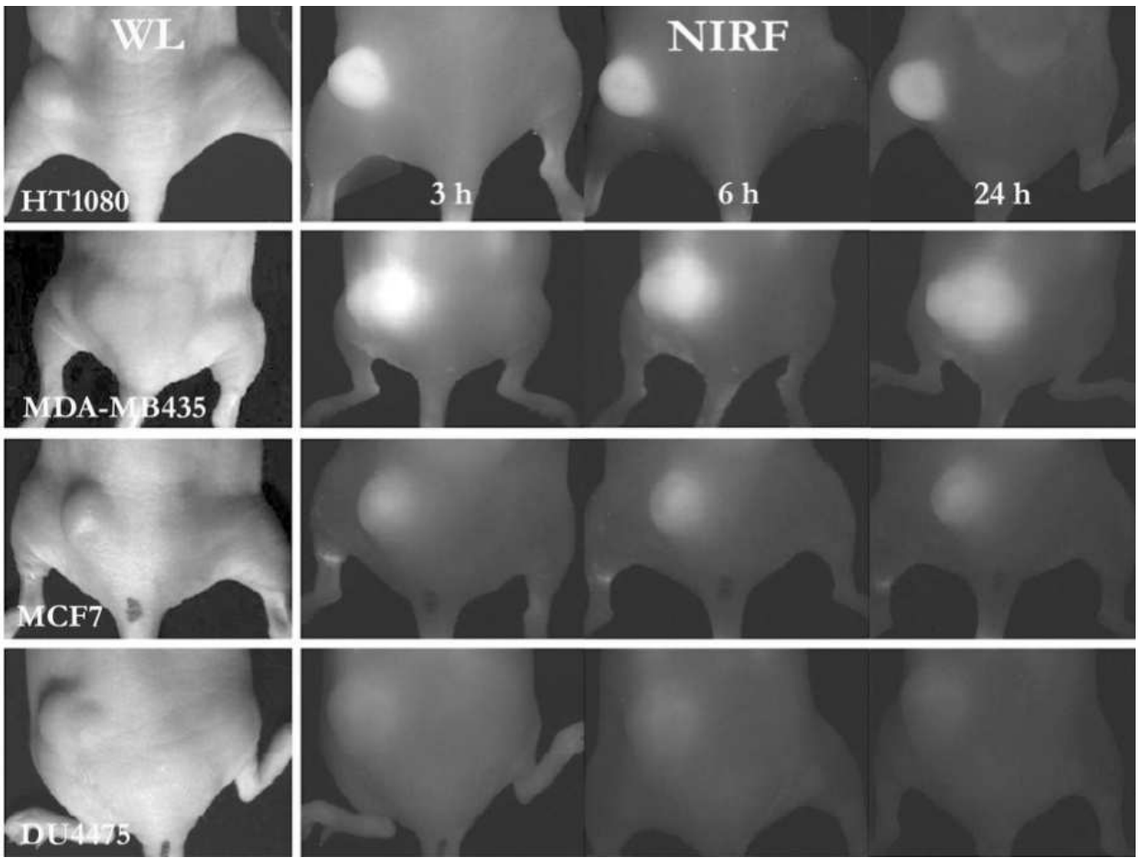

Fig. 4. Comparison of perfusion-type fluorescent dye SIDAG application to differently vascularized tumours. White light images of different tumour xenografts (left row), followed by FRI images taken sequentially after application of 2 µmol/kg body weight SIDAG. There is a strong fluorescence signal in HT-1080 fibrosarcomas and in MDA-MB 435 tumours, whereas MCF-7 and DU-4475 adenocarcinomas exhibit only moderate tumour fluorescence (modified from Wall et al., 2008, courtesy of the author).

Van de Ven et al. evaluated contrast-enhanced DOT, using Omocianine (Bayer Schering Pharma, Berlin, Germany), a novel fluorescent contrast agent, in a first clinical trial of 12 patients suffering from BI-RADS 4–5 breast lesions, using MRI as reference modality and a Philips prototype DOT scanner. They observed dose-dependent enhancement in malignant lesions. The fluorophore allowed for a reliable detection of malignant lesions in the breast (Fig. 5). Limitations of this approach comprised the visualization of lesions close to the chest wall as well as the absence of clear anatomical landmarks for lesion localization (van de Ven et al., 2009, 2010). Just recently, Poellinger et al. in a larger study examined 52 patients with 53 suspicious breast lesions (BI-RADS 4–5) by using the CTLM® system. In this placebo-controlled, dose-escalating trial a detection rate of up to 100% (7 of 7 lesions) after injection of 0.1 mg/kg Omocianine has been reported (Poellinger et al, 2011). Overall, higher detection rates were achieved for larger lesions, at smaller breast sizes, and for cases in which the tumour was located closer to the skin. However, there were also limitations in this study including detection of additional lesions or reconstruction artefacts.

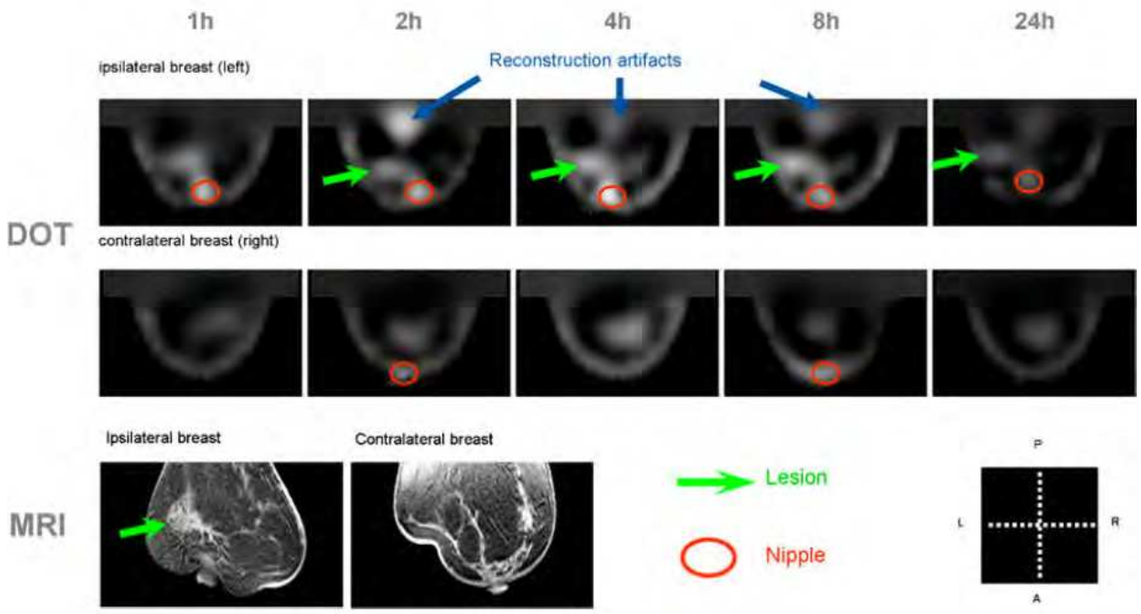

Fig. 5. Contrast-enhanced DOT of the breast: Omocianine-enhanced DOT of a patient with invasive lobular carcinoma in the left breast (first row) with visible contrast agent accumulation in the region of the tumour. The contralateral breast (second row) showed no suspicious contrast enhancement. MRI corroborated the optically detected breast cancer lesions (bottom row) (van de Ven et al., 2010; reproduced with permission from Springer).

In summary, perfusion-type fluorescence contrast agents like ICG, SIDAG or Omocianine (Fig. 6) facilitate a higher target-to-non-target contrast than simple transillumination techniques or non-enhanced DOT. Assuming a linear correlation of signal intensity and local tissue perfusion, contrast enhanced OI could provide quantitative data of tumour vascularization. The future use of these types of contrast agents and imaging procedures in clinical routine, either as an adjunct to or a replacement for x-ray mammography, strongly depends on legal approval and further refinement of this imaging technology.

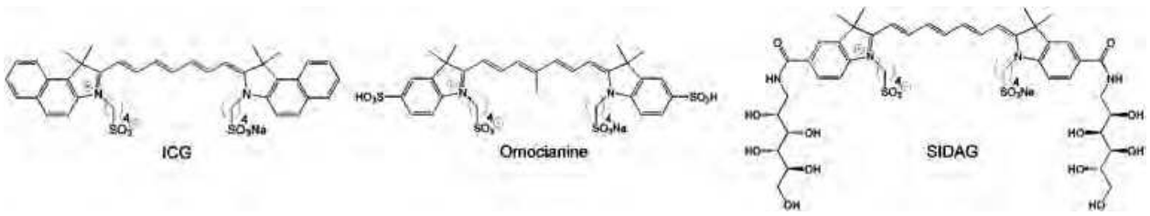

Fig. 6. Molecular structures of ICG, Omocianine and SIDAG.

4. Molecular targets

Breast cancer (BC) is a heterogeneous class of disease, exhibiting a variety of phenotypes and molecular profiles. For selection of the most promising therapy with regard to the molecular profile of a cancer lesion, immunohistochemistry of e.g. biopsies is performed. In this context, identification of e.g. hormone receptor positive (estrogene, ER+, progesterone PR+) or human epidermal growth factor receptor 2 positive (HER-2+) cancers for receptor antagonist driven therapy is frequently possible. Antibody based therapy is routinely combined with classical chemotherapy after surgery for therapy of ER+ and HER-2+ tumours, significantly contributing to therapy success (Alvarez & Price, 2010; Alvarez et al., 2010; Giovannini et al., 2010; Johnson & Brown, 2010; Parker & Sukumar, 2003; Rose & Siegel, 2010). Cancers devoid of the mentioned receptors, so-called triple negative breast cancers (TNBCs), therefore show a relatively poor prognosis due to reduced therapy options (De Laurentiis et al., 2010). Cell surface receptors are, however, not only a promising target for therapy but may also serve for early detection and *in vivo* characterization of breast cancer. Especially growth factors and their receptors, hormone receptors, proteases and integrines have been addressed as targets for diagnostic *in vivo* imaging. This article will give an exemplary overview of successfully performed molecular imaging approaches.

4.1 Growth factors and their receptors

Many cancer cell lines have been shown to produce different types of growth factors, promoting tumour growth and survival. The most prominent of these growth factors is the vascular endothelial growth factor (VEGF). A number of seven different growth factors of this family have been recognized, of which five have been found in human, including placenta growth factor (PlGF), which is responsible for angionenic processes during the menstrual cycle (Fig. 7).

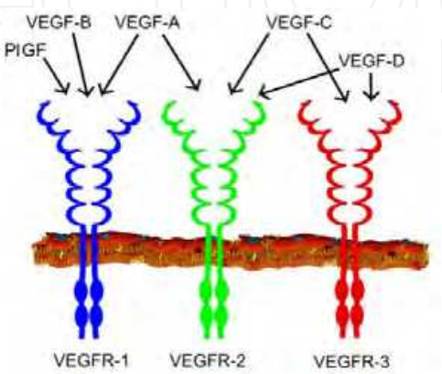

Fig. 7. Human VEGFs and their receptors.

Under physiological conditions, VEGF promotes angiogenesis and vasculogenesis in e.g. wound healing or muscle growth. VEGF expression is to a considerable extent induced by low oxygen supply. Hypoxia inducible factors (HIF-1α and -2α) are the main determinants in this context, inducing VEGF mRNA transcription. Other elements enhancing VEGF expression are intracellular adenosine, interleukin-6 (IL-6) and transforming growth factor β (TGF-β). Signalling of VEGF is mediated by binding of these peptides to three closely related tyrosine kinase (TK) cell surface receptors (VEGFR-1, -2 and -3) which dimerize upon activation and induce endothelial cell (EC) proliferation, vascular remodelling and neovascularization (De Laurentiis et al., 2010; Gasparini, 2000; Giovannini et al., 2010; Guo S. et al., 2010a; Hasan & Jayson, 2001; Hendrix et al., 2000; Hicklin & Ellis, 2005). In patients, elevated expression of VEGF and/or its receptors is often associated with reduced event-free or overall survival. Therefore anti-angiogenic therapy approaches targeting the VEGF system have been developed and tested in first clinical trials. The VEGF system can also be employed for the *in vivo* determination of receptor expression profiles of tumours in preclinical settings. In 2007 Backer et al. facilitated the use of a recombinant single chain protein construct (scVEGF) to image VEGFR expression *in vivo* by OI in combination with single photon emission computed tomography (SPECT) and positron emission tomography (PET). The Cy 5.5 labelled probe (scVEGF/Cy) was used to image human breast cancer cell line MDA-MB 231 and murine mammary tumour cell line 4T1, both transfected for luciferase expression for bioluminescence imaging (BLI), in nude mice. The tracer was shown to bind to tumour vasculature, even in barely palpable lesions, indicating an early influence of the VEGF system in the process of tumour progression. In both tumour models, Cy 5.5 fluorescence could be co-localized with immunofluorescence staining of VEGF receptors and CD31 (Fig. 8) (Backer et al., 2007).

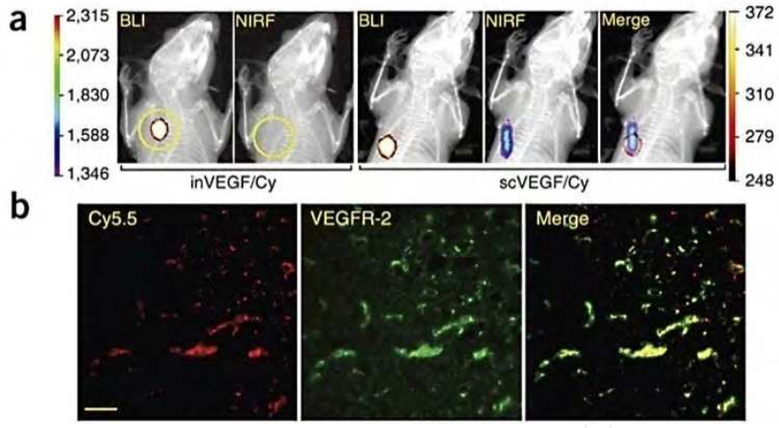

Fig. 8. NIRF imaging with scVEGF/Cy and localization of Cy5.5 on histological sections. (**a**) NIRF imaging and BLI of 4T1luc-tumour-bearing mouse injected intravenously with scVEGF/Cy or inactive control peptide inVEGF/Cy. (**b**) Co-localization of Cy5.5 fluorescence and immunofluorescence staining for VEGFR-2 on 4T1luc tumour cryosections indicates the potency of the CY 5.5 labelled protein conjugate scVEGF/Cy for *in vivo* imaging of VEGF receptors. (Reproduced from Backer et al., 2007 with permission from Nature Publishing Group).

Although the tracer showed a highly heterogeneous accumulation and retention, specific binding and consecutive internalization of the scVEGF-based agent by VEGF receptors in

the tumour area was assumed. Generally, it is recognized that the *in vivo* imaging of VEGF receptors will facilitate assessment of angiogenesis-related parameters for personalized treatment in the future. In another approach, the anti-VEGF monoclonal antibody bevacizumab (Avastin®), a therapeutic against diverse metastatic cancers used in the US since 2004, was labelled with a fluorescent dye and explored for the *in vivo* imaging of VEGF. The scintigraphic imaging of VEGF receptors employing ^{111}In- and ^{89}Zr-labelled bevacizumab conjugates is also discussed as a rapid technique to follow therapy response with SPECT and PET (Nagengast et al., 2007).

Epidermal growth factor receptors (EGFRs) are closely related to VEGF-Rs, sharing their tyrosine kinase activity and their frequent presence on the cell surface of tumours, also promoting proliferation and cell-survival. Trastuzumab (Herceptin®) is a monoclonal antibody against human epidermal growth factor receptor 2 (HER-2). In 2004, fluorescently labelled Herceptin® has been used for the stratification of tumour lesions with regard to HER-2-expression. *In vivo* signals after application of the probe reflected the strong or moderate level of HER-2-expression of human breast cancer cell line SK-BR-3 (high HER-2-expression) or human squamous cell carcinoma cell line PE/CA-PJ34 (mild HER-2-expression) (Fig. 9, Hilger et al., 2004).

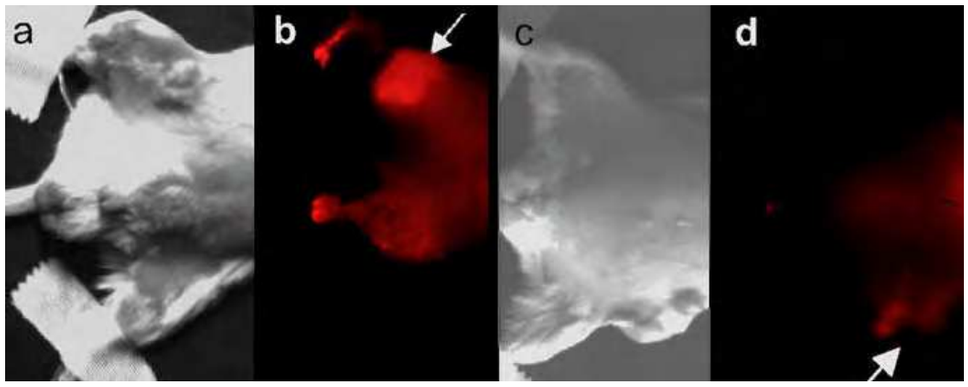

Fig. 9. White light (**a**, **c**) and fluorescence images (λ_{em}=694 nm, **b**, **d**) of xenografted mice after application of 100 µg Cy5.5-labelled Herceptin® in an SK-BR-3 tumour (**a**, **b**) and a PE/CA-PJ34 tumour (**c**, **d**). The highly expressing SK-BR-3 tumour clearly shows a distinct fluorescence signal compared to the normal expressing PE/CA-PJ34 tumour (arrows, modified from Hilger et al., 2004; reproduced with permission from Springer).

Lee et al. have used engineered affibodies for HER-2 imaging ($Z_{HER2:342}$). Affibodies are highly water-soluble α-helical proteins, which can be produced from bacterial systems and have a high affinity for HER-2 besides a much smaller size compared to antibodies (20x) or antibody fragments (4x). In their study, three different types of HER-2-specific affibody molecules were conjugated with AlexaFluor dyes and compared with AlexaFluor–labelled trastuzumab in terms of affinity and specificity to the HER-2 receptor *in vitro* and *in vivo*. The human breast cancer cell line SK-BR-3 was used as positive control, xenografted in nude mice. Interestingly, in addition to the trastuzumab-AlexaFluor conjugate, only the dimeric form of the affibody coupled to an albumin binding domain (ABD) showed accumulation in the tumour region and could be used for the imaging of HER-2 *in vivo* (Fig. 10). Monomeric or simple dimeric forms of the affibody-AlexaFluor-conjugate showed a rapid renal clearance and therefore failed to accumulate in the tumour (Lee et al., 2008).

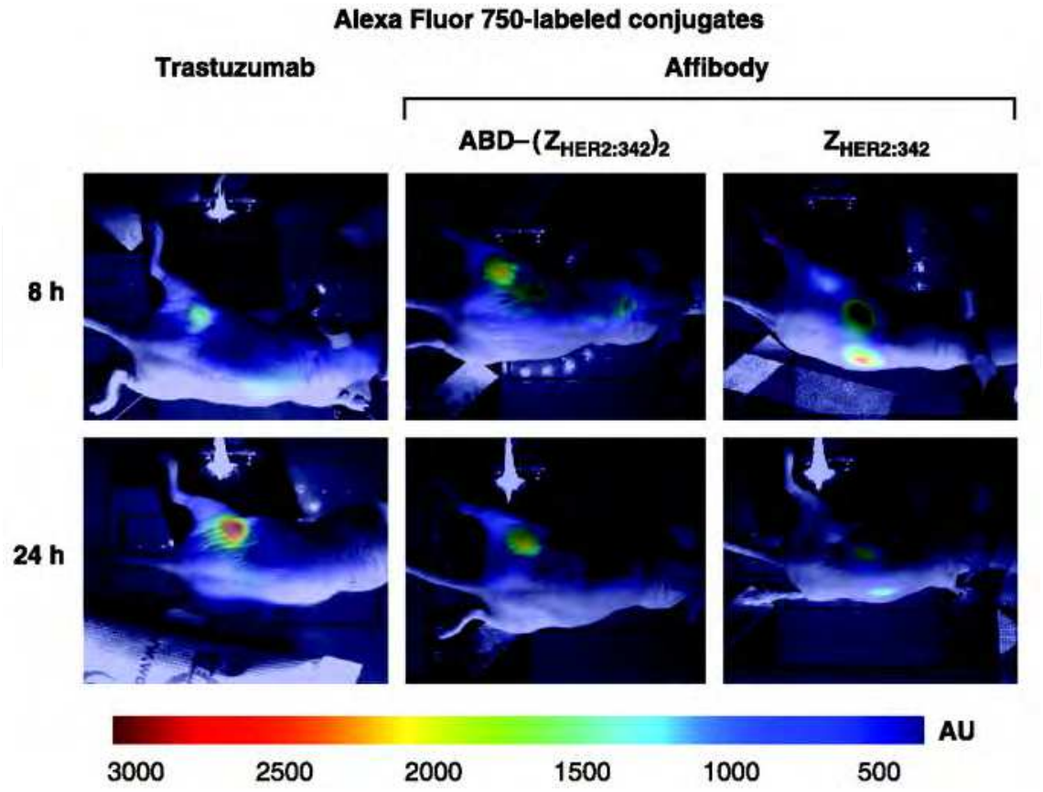

Fig. 10. *In vivo* NIR optical imaging of HER-2-expressing tumour xenografted mice after tail-vein injection of the AlexaFluor 750 labelled conjugates, showing pseudocolor fluorescence images obtained after injecting trastuzumab-AlexaFluor 750, affibodyABD-$(Z_{HER2:342})_2$-AlexaFluor 750, or affibody $Z_{HER2:342}$-AlexaFluor 750 conjugates. Images of trastuzumab-Alexa Fluor 750 conjugate were taken 6 hrs after injection instead of 8 hrs (left column)(Modified from Lee et al., 2008; reproduced with permission from AACR).

Human adenocarcinoma cell lines provide a huge variety of cell surface receptors. MDA-MB 468, e.g. is an EGFR-positive cell line, while MDA-MB 435 is EGFR-negative. These two cell lines have been chosen to evaluate the performance of Cy5.5-labelled EGF *in vivo*. Ke et al. used a custom-made imaging device to examine mice bearing xenografts of these tumour types in the chest walls. A fluorescence signal could clearly be visualized in MDA-MB 468 tumours, but not in MDA-MB 435 tumours. Specificity of binding could be confirmed by blocking experiments with anti-EGFR antibody C225 (cetuximab, Erbitux®) (Ke et al., 2003). A similar approach was used by Wang and Chen in 2009 (Wang K. et al., 2009). They labelled the, by now commercially available, antibody to generate Erbitux®-Cy5.5 as an EGFR imaging tracer. The xenograft model they used was also adenocarcinoma-based. MDA-MB 231 and MCF-7 were chosen as strong and moderate EGFR-expressing cell lines, respectively. Murine xenograft models of both tumour entities were imaged and the tracer was shown to specifically accumulate in tumour regions 24 hours after intravenous injection with the EGFR-overexpressing MDA-MB 231 tumour showing an about two-fold higher fluorescence signal than the only moderately EGFR-expressing MCF-7 tumour (Fig. 11). Again, specificity was proven by excess unlabelled antibody treatment (blocking).

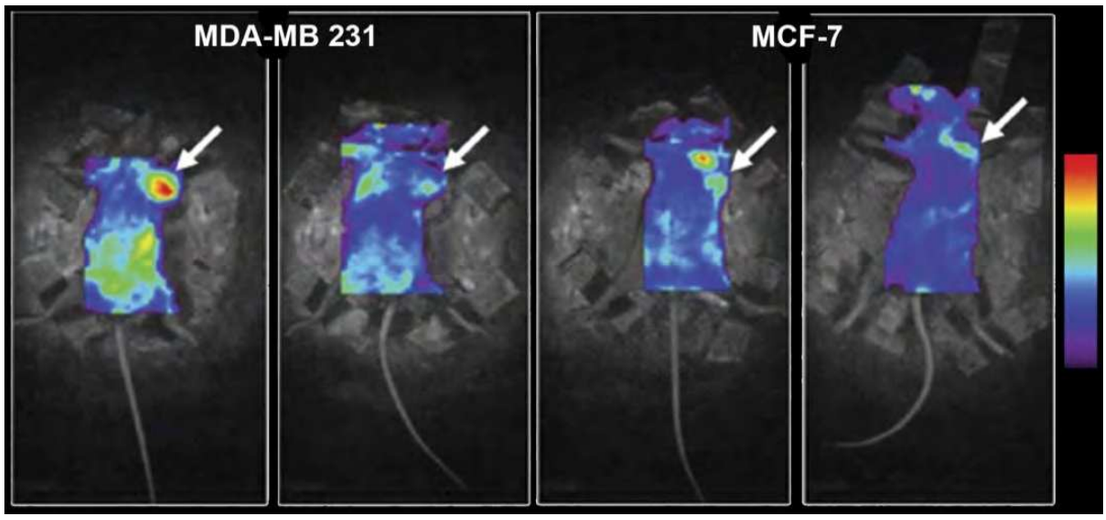

Fig. 11. *In vivo* near-infrared (NIR) images of MDA-MB-231 and MCF-7 xenografted (arrows) nude mice at 24 hours post injection of Erbitux®-Cy5.5. The fluorescence signal is clearly visualized in the left thoracic tumour region of MDA-MB-231 and MCF-7 xenografts. Blocking experiments indicate an apparent decrease of the fluorescent signal by pre-injection of excess Erbitux®, proving specificity (modified from Wang K. et al., 2009; reproduced with permission from InformaHealth).

4.2 Hormone receptors

Estrogen receptors (ERs) are over-expressed in around 70% of breast cancers, which are then referred to as ER-positive or hormone-dependent. Tamoxifen is the most frequently used anti-hormonal drug for treatment of women with hormone-dependent breast cancer. Tamoxifen treatment is very effective in these cases and significantly reduces the mortality of breast cancer patients. Unfortunately, during treatment many tumours develop a resistance to the drug, often accompanied by a reduction or loss of estrogen receptors. Several mechanisms have been discussed to be responsible for this development (Kumar 2007) and a non-invasive imaging technology to detect ER expression *in vivo* could help identifying the underlying process. A recent approach from Jose and co-workers describes the labelling of estradiol with a carbocyanine dye for the evaluation of the hormone receptor status *in vivo* and could therefore help in decreasing the need for unnecessary biopsies. Future research will show the performance of the developed tracer in preclinical models (Jose et al., 2011).

A rather particular system in this context is represented by the endothelin (ET)-axis, which is comprised of the three peptide hormones ET-1, ET-2 and ET-3 and their two associated receptors ET_AR and ET_BR. Endothelin (ET) was first described as a 21-amino acid peptide with vasoactive potency; in fact ET-1 is one of the most vasoconstricting substances currently known (Hickey et al., 1985; Yanagisawa et al., 1988). In addition to its role as a vasoconstrictor, more recently the role of the ET-axis as a progression factor in certain human cancers, including breast and ovarian cancer, has been discussed (Bhalla et al., 2009; Rosano et al., 2010). The influence of the ET-axis is attributed to elevated levels of ET-1 as well as overexpression of ET receptors on tumour cells and tumour associated cells (e.g. fibroblasts, endothelial cells, macrophages). In addition, a close interaction of the ET-axis

with EGFR/HER-2 signalling (Fischgräbe et al., 2009) and VEGF-induced angiogenesis (Kandalaft et al., 2010) has been found in breast cancers and consequently ET receptor antagonists as new chemotherapeutic drugs for cancer therapy have been introduced. The evaluation of the ET receptor status *in vivo* would allow for early diagnosis and clarify the impact of e.g. anti-angiogenic therapies. In 2007 our group reported the design of a small-molecular ET_AR antagonist conjugated to a fluorescent dye via a short polyethylene glycol spacer (Fig. 12), capable of imaging tumour-associated ET_AR-expression *in vivo* (Höltke et al., 2007; Höltke et al., 2009).

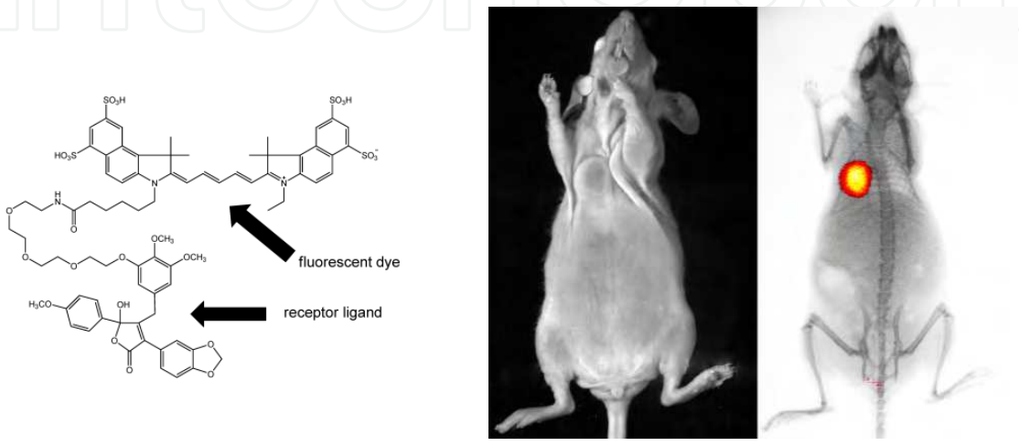

Fig. 12. Molecular structure of the designed fluorescent ET_AR tracer (left). White light (middle) and merged x-ray and fluorescence (FRI) image (right) of tumour-associated ET_AR expression in a murine breast cancer xenograft model 24 hrs after i.v. tracer administration (Höltke, unpublished results).

4.3 Integrins

Integrins are cell-adhesion proteins that mediate cell-cell and cell-extracellular matrix interactions, thereby increasing tumourigenicity and invasiveness of cancer. They are composed of an α and a β subunit. So far 24 α/β combinations (αβI) have been identified, seven of them have been demonstrated to bind the RGD motif, an arginine-glycine-aspartic acid-based peptide sequence (Arnaout, 2002; Arnaout et al., 2002; Hood et al., 2003; Schottelius et al., 2009). Also, the two αβI known to be most active in tumoral neoangiogenesis, the vitronectin receptor αvβ3 and the fibronectin receptor α5β1, bind RGD with high affinity. Integrin αvβ3 is a transmembrane protein, which is specifically expressed on tumour surfaces and on activated and proliferating endothelial cells. In 1998, Gasparini and co-workers introduced vascular αvβ3 as a prognostic factor in breast cancer (Gasparini et al., 1998). Soon after first RGD-based radiotracers for PET or SPECT were developed (Choe & Lee, 2007; Liu, 2006) fluorophore-labelled RGD derivatives were designed for NIR optical imaging approaches (Chen et al., 2004; Cheng et al., 2005; Gurfinkel et al., 2005; Ye et al., 2006). Since linear peptides containing the RGD sequence provide only low integrin subtype selectivity and are furthermore rather unstable under metabolic conditions cyclic RGD peptides were synthesized, which show a higher selectivity for αvβ3 and are characterized by a sufficient metabolic stability (Fig. 13).

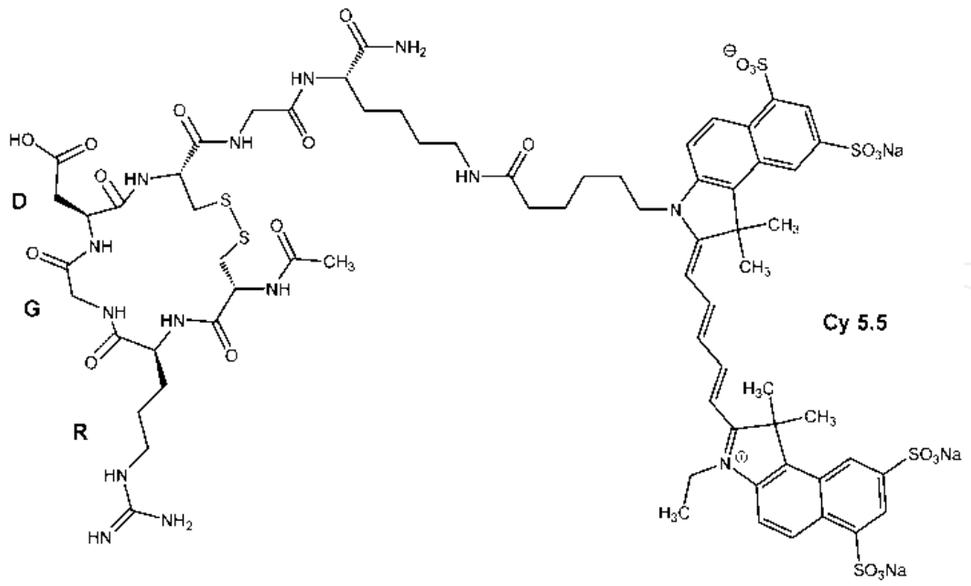

Fig. 13. Molecular structure of a typical cyclic RGD peptide conjugated to a fluorescent dye.

First experiments with Cy 5.5-labelled RGD peptides were published in 2004 (Chen et al., 2004; Wang W. et al., 2004) and aimed at glioblastoma, sarcoma and melanoma tumour entities. Besides the peptidic αvβ3 ligands derived from RGD, antibodies, nanobodies and small molecular peptidomimetics have been presented (Winter et al., 2003; Winter et al., 2006; Xie et al., 2008). In 2007, von Wallbrunn et al. visualized αvβ3 activity in three different xenograft models, including breast cancer, using FRI and FMT (von Wallbrunn et al., 2007). Both modalities provided mutually correlating results, confirming histological gold standard measurements (Fig. 14, left). Recently, Mulder et al. presented an RGD-conjugated quantum dot (QD) for visualization of αvβ3 expression in melanoma-bearing mice (Mulder et al., 2009). The QDs were coated with gadolinium diethylene-triaminepentaacetic acid (Gd-DTPA)-based lipids and therefore serving as bimodal imaging agents for a use in MRI and OI. *In vivo* examinations revealed an attachment of the QDs to tumour vessels after i.v. injection. Interestingly, a labelling of blood vessels as far as 1 cm outside the primary tumour lesion was observed, indicating activation of distant vascular endothelium. Confirming the specificity of binding to tumour vasculature, no significant accumulation of QDs in distant vessels of e.g. muscle was observed. The combination of MRI and fluorescence imaging allowed both exact anatomical localization and evaluation of angiogenic activity. In this study, luciferase-transfected tumour cells were used, providing bioluminescence images of the tumour for additional correlation of the QD-triggered imaging findings (Fig. 14, right).

In summary, a large number of integrin-targeted optical probes have been developed and tested. Most approaches base on RGD-containing small peptides labelled with a fluorophore or conjugated to fluorescent nanoparticles (quantum dots). Many of these show promising results concerning sensitivity and specificity for tumour vasculature, demonstrating the potential of integrin targeting in cancer imaging. A representative review paper on the diverse structures of integrin targeted optical probes has recently been published, also touching the potential of integrin-based therapy (Ye & Chen, 2011).

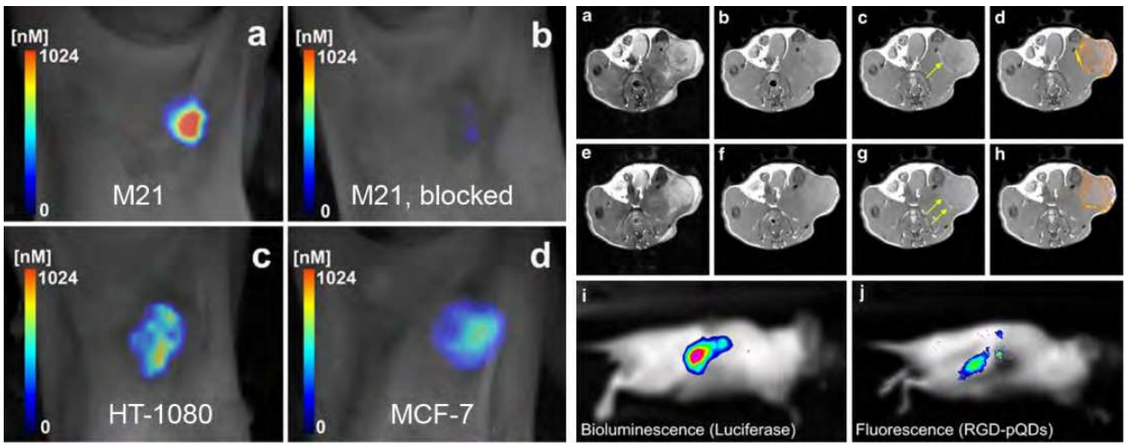

Fig. 14. Two examples of recent integrin imaging approaches. Left: Fluorescence mediated tomography (FMT) images of different tumour entities 60 minutes after injection of a Cy 5.5-labelled cyclic RGD peptide into athymic nude mice (2 nmol/animal), displaying tracer accumulation in the tumour lesion dependent on target structure expression and predosing (modified from von Wallbrunn et al., 2007, courtesy of the author). Right: MRI and OI of tumour-related angiogenesis visualized by αvβ3 integrin-targeted multimodal QDs (pQDs): MRI, bioluminescence and FRI of a C57B16 tumour-bearing mouse before and after injection of RGD-pQDs. T2-weighed MR images (a,e) show the contour of the tumour on the flank. T1-weighted images were measured before (b,f) and 45 min after (c,g) the injection of the RGD-pQDs. The arrows in (c,g) indicate bright (positive contrast) regions in the periphery of the tumour. In d and h strong signal enhancement is colour-coded (red-orange). Correlative FRI (j) shows good co-localization of the signal with bioluminescence (i) of the tumour cells (reproduced from Mulder et al., 2009 with permission from Springer).

4.4 Proteases

Matrix metalloproteinases (MMPs) are closely connected to the pathophysiological properties of integrines, which also makes them relevant factors associated with tumour growth, progression and metastasis (Brooks et al., 1996; Chabottaux & Noel, 2007; Stefanidakis & Koivunen, 2006). Human MMPs are a family of 24 structurally related zinc ion-dependent endopeptidases, able to degrade almost all components of the extracellular matrix (ECM) and the basal membrane. Moreover, they contribute to the morphogenesis of endothelial cells and have been shown to regulate the transcription of growth factors. The large number of MMPs and their diverse routes of involvement in cancer progression have made it difficult to address their activity in therapeutic interventions. First clinical trials with broad-spectrum MMP inhibitors like *Marimastat* or *Batimastat* were discontinued because of poor performance and severe side effects (Dorey, 1999; Renkiewicz et al., 2003). In this respect, especially the gelatinases MMP-2 and MMP-9 were identified as diagnostic factors in breast cancer on both, their local and systemic level, frequently predicting lymph node involvement and metastatic status (Jezierska & Motyl, 2009; Radisky E.S. & Radisky D.C., 2010). The *in vivo* assessment of MMP activity would be highly desirable for diagnosis and the evaluation of therapy. Bremer et al. have developed a fluorescently labelled substrate probe for MMP-2 (Bremer et al., 2001a; Bremer et al., 2001c). A graft copolymer, consisting of a poly-lysine backbone and polyethylene glycol side chains, was equipped

with Cy 5.5 labelled specific peptidic substrates cleavable by MMP-2. Due to the close proximity of the dyes their fluorescence is quenched by the transfer of electronic excitation energy between two (or more) molecules (fluorescence resonance energy transfer, FRET) (Kiyokawa et al., 2006). Upon cleavage of the peptidic substrate by MMP-2 the dye molecules are removed from the backbone polymer, their fluorescence is dequenched and a signal can be detected. The resulting signal to background ratio is unrivalled due to virtually absent background fluorescence. (Fig. 15).

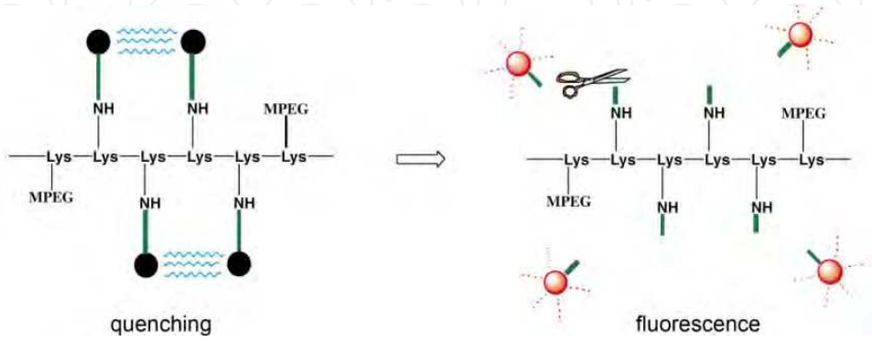

Fig. 15. Principle of the use of FRET for the detection of proteolytic activity. When dye molecules are in close proximity to each other, the fluorescence is quenched by FRET (black circles). After cleavage of the peptide substrate by e.g. MMP-2 (scissors) fluorescence can emerge (red circles). Modified from Bremer et al., 2001a, courtesy of the author.

The probe was applied to xenograft models of human fibrosarcoma HT-1080 and human breast adenocarcinoma BT-20. While HT-1080 cells express a high amount of MMP-2, BT-20 cells show only moderate protease levels. Accordingly, *in vivo* examinations show strong fluorescence signals from HT-1080 tumours, compared to only low signals from BT-20 xenografts (Fig. 16).

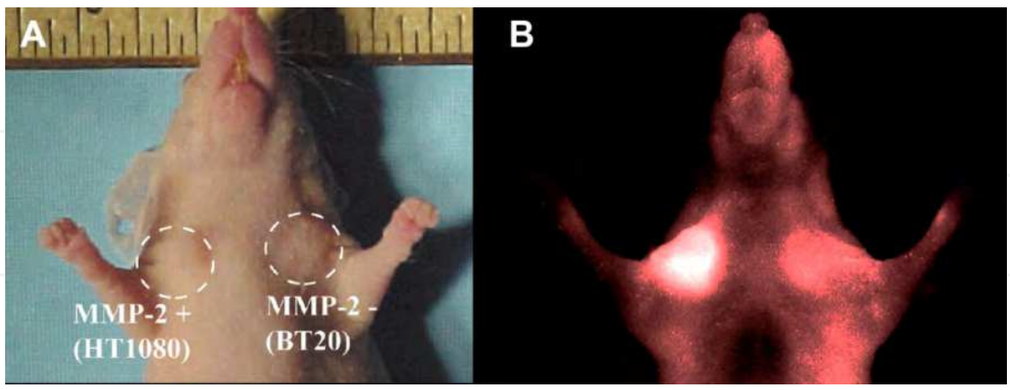

Fig. 16. Demonstration of protease activity by NIRF imaging in murine xenograft models of MMP-2 positive human fibrosarcoma HT-1080 and MMP-2 negative adenocarcinoma BT-20. A: Colour photograph of the implanted nude mouse. Both tumours measured approximately 2 x 3 mm. B: The NIRF image shows that the fibrosarcoma generated strong fluorescent signal intensity 2 hours after i.v. injection of the MMP-2–sensitive probe, but the signal intensity of the BT-20 tumour was only slightly higher than background fluorescence (modified from Bremer et al., 2001a, courtesy of the author).

The method has also been successfully applied for the imaging of cathepsins (Bremer et al., 2002). Cathepsins represent another group of proteases involved in cancer progression and the growth of metastases. Like MMPs, cathepsins comprise a large group of proteins responsible for a variety of processes in the animal/human body. Unlike MMPs, cathepsins do not depend on a Zn-atom at the active site, but are serine, cysteine or aspartate dependent. Most cathepsins are lysosomal proteins, activated by the low pH value inside the organelle. In particular cathepsins B, D, K and L2 have been assigned a role in breast cancer development and metastasis (Duffy, 1996; Nomura & Katunuma, 2005; Radisky E.S., 2010; Rose & Siegel, 2010). In the presented examination, a cathepsin B sensitive probe was designed and used to distinguish a highly invasive (DU-4475) and a well differentiated adenocarcinoma (BT-20). The less invasive tumour showed a significantly lower fluorescence signal (- 35%) than the highly invasive one (Bremer et al., 2002).

5. Conclusion

The successful preclinical application of optical imaging methods for cancer imaging has been extensively demonstrated over the last years. Especially in small animal imaging, techniques for tumour characterization (e.g. surface target expression or vascularization) have been developed and validated. The synthesis of specific tracers for targets arising from the ever-growing pathophysiological understanding of diseases can easily be performed and does not require large-scale laboratory equipment. This enables more and more scientists to accelerate their research on targeted *in vivo* imaging. In addition, OI devices offer quick and convenient image acquisition and reconstruction, especially compared to other small animal imaging technologies. In basic research, OI of breast cancer is a well established part of everyday work. Future use of OI in clinical routine depends on several factors, amongst which probe design and approval and further improvement of the imaging techniques appear the most important. For the elaboration of both factors, industry – either pharmaceutical- or medical technology companies – is required to partner with academic research institutions. On the technical side, spatial resolution and access to deep tissue lesions are the most striking and obvious points, where the refinement of techniques has to focus on. Pioneering work in this area is accomplished by the companies already distributing optical breast scanners. First trials with smaller groups of patients showed promising results, although by today the technique has to be considered an adjunct to conventional x-ray mammography only. On the field of tracer development, simple perfusion tracers, targeted fluorescent ligands, protease-sensitive smart probes and fluorescent nanoparticles or a combination of these still struggle for highest diagnostic accuracy on the one hand versus applicability on the other. It remains to be seen, whether targeted imaging will make the way into clinical routine and which role OI can play in this context. From the vast variety of targets relevant for basic research imaging, the identification of few molecular markers, expressed by a wide range of cancer entities seems to be a promising first step towards targeted molecular imaging, especially with regard to the immense regulatory effort and major investments (toxicology studies, GMP-production facilities etc.), which approval of a contrast agent requires. The existence of ICG on the market, though, substantiates the general possibility and shows that there is a demand for optical imaging contrast agents in the clinics and an increasing interest for further development in this field of alternative imaging technologies.

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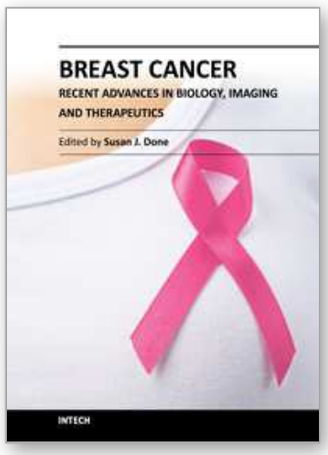

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In recent years it has become clear that breast cancer is not a single disease but rather that the term encompasses a number of molecularly distinct tumors arising from the epithelial cells of the breast. There is an urgent need to better understand these distinct subtypes and develop tailored diagnostic approaches and treatments appropriate to each. This book considers breast cancer from many novel and exciting perspectives. New insights into the basic biology of breast cancer are discussed together with high throughput approaches to molecular profiling. Innovative strategies for diagnosis and imaging are presented as well as emerging perspectives on breast cancer treatment. Each of the topics in this volume is addressed by respected experts in their fields and it is hoped that readers will be stimulated and challenged by the contents.

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