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TOPICALREVIEW

Integrating imaging, exosome and protein network rewiring information to track early tumour evolution of resistance mechanisms

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KEYWORDS: Exosome, FRET, FLIM, Dimerisation, HER family, Oncogenic rewiring, Drug resistance

Abstract

Despite recent advances in developing HER-family targeted drugs, clinical trials have shown poor results. Tumour evolution takes place overtime, frequently leading to aberrant new signalling cascades that disrupt the efficacy of targeted therapies and ultimately cause patients to develop resistance against initially effective drugs. To predict outcome and stratify treatment there is an imperative need to develop a systems understanding of concentration- independent parameters that could be monitored in trials and report on tumour evolution. Amongst the circulating tumour markers, exosomes offer a suitable platform for the longitudinal monitoring of protein network signalling in the form of a liquid-biopsy by imaging receptor dimerization status. Here, we illustrate the biomarker utility of monitoring oncogenic receptor signal rewiring using exosomal FRET/FLIM to aid the prediction of clinical outcome and patient treatment stratification.

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1. Introduction

An increasing number of drugs and treatments against cancer have shown high efficacy and promises *in vitro* with recent technological developments. However, many of these promising agents showing low primary resistance were presented with poor long term results in the clinic due to acquired secondary resistance, leading to inevitable disease relapse and a dramatic reduction of patients' survival. The acquired resistance is one of the main hurdles for the drug treatment efficacy. Recent studies have not only shown high inter-tumour heterogeneity between different patients of the same cancer types, but also significant intra-tumour heterogeneity within the same patient. This heterogeneity is caused by clonal evolution of the tumour, whereby mutational changes give rise to many different subpopulations of the tumour [1-3]. This complex and somewhat unpredictable tumour evolution process complicates the efficacy of long term treatment, as resistance easily develops against single-targeted cancer therapies. Moreover, the individual heterogeneity makes the stratification of personalised medicine extremely difficult [4, 5]. Thus, being able to understand and monitor the underlying mechanisms of acquired resistance remains very important for the clinic [6].

Currently, the clinic mainly uses information obtained from tissue immunohistochemistry combined with imaging techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET) for the purpose of prognosis and choosing therapy for the patients. However, with the rise of the *-omics* era and the continuous emergence of state-of-the-art techniques, there is now more information at the clinicians' disposal to make much better informed decisions. Amongst the many emerging biomarkers, a type of cell-derived extracellular vesicles found in almost all bodily fluids called exosomes have much to offer. They have been implicated by many to play a role in cancer [7-11], and they also provide clinicians with a minimally invasive method of obtaining valuable information via liquid biopsy. Cell surface receptors are also found on the surface of exosomes, and many studies have shown the role of these cell surface receptors in acquired resistance [12-16].

The focus of this review will be on how resistance is acquired against HER (Human Epidermal growth factor Receptor) family targeted drugs, the role played by the individual receptors and the importance of using HER family receptor dimerization status as a means to track early tumour evolution development. As an example, we show our results in patient cancer tissue samples as previously reported [1] and suggest expanding this approach to patient serum exosomes which have the additional advantage of easily allowing the longitudinal monitoring of the tumour.

2. HER Family and cancer

The HER (Human Epidermal growth factor Receptor) family are RTKs (Receptor Tyrosine Kinase) that include EGFR (Epidermal Growth Factor Receptor) (also known as ErbB1 or HER1), HER2 (Erb2), HER3 (Erb3), and HER4 (Erb4), all of which participate in regulation of cellular homeostasis [17]. Each member of the HER family plays key roles in many cellular processes such as proliferation, differentiation, migration, and cell death programming. With the exception of HER2, other HER family members are inactive prior to ligand binding. These transmembrane receptors will dimerize upon activation by ligand binding on the extracellular domain, forming homo- and heterodimers. Receptor dimerization causes a transactivation of the intracellular domain by trans-autophosphorylation, which is the first of a series of signal transduction events. This leads to the recruitment of intracellular molecules, and activates the 4 main intracellular pathways downstream of the HER family: Ras/Raf/MEK/MAPK, PI3K/Akt, JAK/STAT, and PLC γ /PKC. These signalling pathways regulate a wide range of intracellular events including proliferation, differentiation, apoptosis, migration, adhesion, gene expression, cell cycle progression, transcription, translation, and angiogenesis.

Disease progression is often favoured when the tightly regulated and highly complex interactions between these proteins are altered. In cancers, these pathways trigger hallmarks of cancer: apoptosis inhibition, survival, proliferation, loss of cell cycle control, invasion, and metastasis. Changes in EGFR - upregulation, mutation, overexpression, translocation - and EGFR signalling, are frequently found in many different cancers such as brain, breast, ovarian, cervical, colorectal, gastric, melanoma, head and neck, as well as pancreatic cancers, and are often associated with poor prognosis and survival. HER2 has long been identified as an important biomarker for a major subtype of breast cancer that is overexpressing HER2, and is routinely being used in the clinic as a biomarker for therapeutic decisions [18]. Apart from gastric [19], prostate[20], and ovarian cancers [21], recent studies have also suggested the potential of HER2 as a therapeutic target in other cancers, such as biliary tract cancer[22]. In cancers of epithelial origin such as breast, ovarian, cervical, colorectal, gastric, melanoma, head and neck, and pancreatic cancers, HER3 overexpression has been observed and is associated with poor prognosis. HER4 has been gaining increasing attention in the recent years, where its expression profile is implicated to be important in several cancers such as breast [23, 24], gastric[25], rectal [26], and lung [27].

The apparent causative link between the HER family and tumourgenesis is the reason why EGFR and the HER family have been studied vigorously for decades and remains one of the most valuable therapeutic targets since the discovery of EGFR.

3. Oncogenic rewiring

The key roles that the HER family play makes them obvious therapeutic targets for anti-cancer therapies. Unsurprisingly, the current standard clinical practice for stratifying cancer patient treatment is based on the expression level of HER family members, depending on the cancer type [17, 26]. To date, all FDA approved cancer therapies in the clinic targeting the HER family members can be divided into 2 classes [3]. First, there are small molecule TKIs (tyrosine kinase inhibitors). They inhibit phosphorylation of RTK (Receptor Tyrosine Kinase) domain either by competitive binding or allosterically by locking the RTKs in a non-functional/inactive conformation. Examples of TKIs include Erlotinib (EGFR inhibitor), Gefitinib (EGFR inhibitor), Lapatinib (EGFR & HER2 dual inhibitor), Osimertinib (EGFR inhibitor), and Vandetanib (EGFR inhibitor). The other class of targeted therapy is represented by blocking antibodies. These mAbs (monoclonal antibodies) act by directly binding to the extracellular domains of RTKs, causing blockage to RTK activities and therefore trigger apoptosis. Drugs of this class include Cetuximab (EGFR mAb), Panitumumab (EGFR mAb), Nectinumab (EGFR mAb), Pertuzumab (HER2 mAb), and Trastuzumab (aka Herceptin, HER2 mAb). These drugs have been very effective in many cancers in the clinic, however many patients develop resistance against these therapies often within one year or less [28, 29].

The principle of all targeted therapies is based on the interruption of pro-oncogenic signalling cascades. However, many of these pathway interventions can be disrupted by cancer-induced changes, resulting in the formation of new, ectopic signalling that favours tumour development. This acquired resistance is known as oncogenic rewiring [30], and is one of the main reasons for the failure of targeted therapies against the HER family in the clinic [16]. This principle of oncogenic rewiring has led to a successful implementation of anti-HER therapeutic, multi-targeted combination in preclinical cancer models [31]. In clinical trials many cases of initial responders eventually become resistant by developing secondary (or acquired) resistance. Several mechanisms were proposed to explain the oncogenic rewiring of the tumour leading to the acquisition of resistance against HER family targeted therapies. Mutations in the RTK's extracellular domain could prevent inhibitory binding by targeted mAb therapies while those in the intracellular kinase domain could interfere with TKIs inhibitory functions. Mutations activating downstream effectors of the targeted RTK lead to consistent active signalling despite inhibition of the receptor's kinase activity. Activation of an alternative signalling pathway could lead to a compensatory shift in the dependence of the survival signalling away from the target under therapeutic pressure. All of the above support the existence of an escape mechanism by which tumour can resist and evade targeted therapies.

EGFR, being the target of many clinical drugs, displays oncogenic rewiring through different ways. In lung cancer, EGFR can develop a secondary, acquired T790M mutation, which is commonly found in resistant patients [12]. Besides receptor mutations, there is also

aberrant activation of downstream pathways such as MAPK amplification [32], as well as shifting the pro-oncogenic signalling into other pathways including c-MET [33].

In colorectal cancer in particular, HER2 activation has been shown to cause acquired resistance against Cetuximab, an EGFR-targeted antibody therapy [34]. This is particularly noteworthy in the process of oncogenic rewiring in the HER family, and has been implicated as a major cause for the development of such resistance as pointed out by several studies [15, 28, 33, 35, 36]. Moreover, HER2 amplification has been suggested to assist the acquisition of resistance against anti-EGFR TKIs [37]. HER3 is known to be a 'pseudokinase' because it lacks a functional intracellular tyrosine kinase domain for intrinsic kinase activity, and is therefore entirely dependent on its heterodimerization partners and ligand binding for all downstream signalling [38]. Known dimerization partners of HER3 include EGFR, HER2, HER4, and c-Met. The pseudokinase HER3 has more tyrosine kinase residues on its intracellular C-terminus than the other HER family members, and is therefore able to directly bind to the PI3K subunit p85 for activation of the PI3K/Akt signalling pathway [17, 38]. In particular, the HER2/3 dimer is known to be the most potent oncogenic activator of the PI3K/Akt pathway [39]. As the key regulator of proliferation, the PI3K/Akt signalling cascade is often found to be activated in drug-resistant tumours [1], suggesting the highly pivotal role of HER3 in reducing the efficacy of targeted treatments against the HER family. There are many examples of HER3 oncogenic rewiring in the literature. The activation of HER3 signalling by c-Met has been shown to induce resistance against the EGFR TKI Gefitinib in lung cancer, where the Gefitinib-induced inhibition of EGFR-HER3 signalling pathway lead to the prevalence of HER3 activation by c-Met [33]. A different study showed that resistance to erlotinib in lung cancer patients involves HER2/3 feedback loops which stimulated the extracellular signal-regulated kinase (ERK) pathway, induce transcription of HER2 and HER3 and stimulate the interaction between MET and HER3 [31]. The mechanisms of interaction between c-Met, the epidermal growth factor receptor family and other cell surface protein families, how these contribute to signalling crosstalk, oncogenesis, and drug resistance have been excellently reviewed [13]. The compensatory shift from HER2/3 cascade to EGFR/HER3 cascade under the treatment pressure by pertuzumab [40], alternative HER3 phosphorylation leading to evasion of EGFR and HER2 TKIs [29], HER3 expression-driven resistance against trastuzumab [14], the heregulin β 1 (HRG)-activated HER3 that leads to resistance against lapatinib [41]. Thus, it is of great clinical interest to detect, prevent, and reverse the aberrant activation of new HER3 signalling cascades which lead to intensified efforts to pharmacologically target it [42]. Despite the increased attention towards this particular member of the HER family, no HER3-targeting drugs have been approved for use to date. The development of anti-HER3 therapies (such as monoclonal anti-HER3 antibody therapy) is still facing difficulties due to a lack of established biomarkers for the stratification of anti-HER3

therapy [43]. It has been suggested that HER3 expression level is not the only important biomarker to be taken into consideration during cancer treatment, but also the interactions of this pseudokinase with the other RTK heterodimerization partners [35]. To measure changes in the heterodimer quantities with treatment and monitor the tumour longitudinally we have developed a FRET/FLIM assay as detailed in the following section.

4. Dimerization measurement by FRET/FLIM

A significant body of our previous work supported the idea that the HER dimerization status may be more important than HER receptor expression per se in determining sensitivity or resistance to a given therapeutic agent. The extent of HER dimerization between any two receptor pairs could be quantified by Fluorescence Resonance Energy Transfer (FRET) between a pair of suitable FRET fluorophores directly conjugated to the primary antibodies.

FRET is the radiationless transfer of energy between the dipole of a fluorophore called the donor (D) to the dipole of a fluorophore called the acceptor (A). The transfer efficiency depends on the spectral overlap of the fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor, the relative orientation of the two fluorophores and their separation distance. The FRET efficiency varies with the inverse 6th power of the distance between the donor and acceptor and is typically negligible for distances greater than 10 nm.[44]

The spatial scale on which FRET occurs is compatible with the protein dimension which renders FRET as a powerful tool to probe intra- and inter-molecular distances.[45] If one of the HER family members is labelled with a donor conjugated primary antibody and another HER receptor is labelled with an acceptor conjugated primary antibody, the detection of FRET yields proximity information interpreted as heterodimerization.

To identify and quantify FRET we used Fluorescence Lifetime Imaging Microscopy (FLIM). The fluorescence lifetime refers to the average time the molecule stays in its excited state before emitting a photon, which is an intrinsic property of a fluorophore. Fluorescence lifetime is sensitive to the local environment including pH, refractive index, temperature and insensitive to change in concentration and laser excitation intensity. Time-resolved measurements can distinguish between effects due to FRET or probe concentration. For example, a low donor fluorescence intensity can be caused by either a low donor concentration or efficient quenching by FRET—but only in the latter case is the fluorescence decay shortened. Measurements of the fluorescence lifetime are less susceptible to artefacts arising from scattered light, photobleaching, non-uniform illumination of the sample, light path length, or excitation intensity variations compared to fluorescence intensity measurements. [46]

FRET/FLIM measures the fluorescence lifetime of the donor in the absence and presence of the acceptor and allows for the distinction between average FRET efficiency and FRET sub-population, independent of the local concentration and stoichiometry of donor and acceptor. If the stoichiometry is not known, i.e. the sample contains both interacting and non-interacting donors, then a bi-exponential donor fluorescence decay would result. The non-interacting donors do not undergo FRET, and thus emit fluorescence with the lifetime of the unquenched donor. The donors undergoing FRET exhibit shortened fluorescence decay. The ratio of the preexponential factors, or amplitudes, of the bi-exponential decay represents the ratio of interacting donors undergoing FRET to those not interacting[47].

FLIM can be based on various optical and electronic principles.[48] We have developed a custom-built inverted microscope system operating in the single-photon excitation regime as described elsewhere[49].The FLIM images were analysed to produce distinct lifetimes or a distribution of lifetimes across the image depending on the number of collected photons and the exponential model used. The donor fluorescence was fit to a multi-exponential decay using in-house written software (TRI2) utilising a Levenberg-Marquardt algorithm. Pseudo-colour fluorescence lifetime maps were also produced using TRI2.

Previously, we applied our FRET/FLIM technique to assess various HER receptor dimerization in different cancer cell models, xenografts, FFPE tissues and recently to circulating exosomes from cells, xenograft models and patients as detailed in the next section. We have shown in MCF7 breast cancer cells that erlotinib and gefitinib, but not lapatinib induce EGFR homodimerization[50]. Also, we have demonstrated in the same cell model the existence of EGFR-HER4 dimers and their importance to cell motility [51]. In Hcc1954 breast cancer cells we have shown that gefitinib treatment leads to enhanced EGFR homodimerization[52]. Finally, our aim was to apply this assay to tumour samples in order to assess the prognostic significance and predictive value of HER receptor dimerization in various cancers. In the first clinical application of this technique we quantified EGFR-HER3 dimerization in FFPE tissues from basal-like breast cancer patients treated with a neoadjuvant anti-EGFR treatment (cetuximab or patritumab) and suggested that increased HER3 expression and HER3 activation may mediate residual tumour growth after EGFR-targeted therapy [1]. Figure 1 shows an illustration of the tumour microenvironment (Fig. 1A) and the labelling scheme used to detect EGFR-HER3 FRET (Fig. 2B). The EGFR and HER3 receptors were labelled with fluorescently conjugated cytoplasmic primary antibodies Alexa 546 F4 (EGFR) and Cy5 2F12 (HER3). The fluorescence lifetime of the donor is measured in the absence and in the presence of the acceptor and the FRET efficiency is calculated according to the equation $E_{\text{FRET}} = 1 - \tau_{\text{DA}}/\tau_{\text{D}}$. Lifetime images are reconstructed pixel by pixel and pseudo colored. Representative FRET/FLIM images of the FRET efficiency in two distinct TMAs before (low FRET efficiency shown as dark blue in the donor lifetime pseudocolor map) and

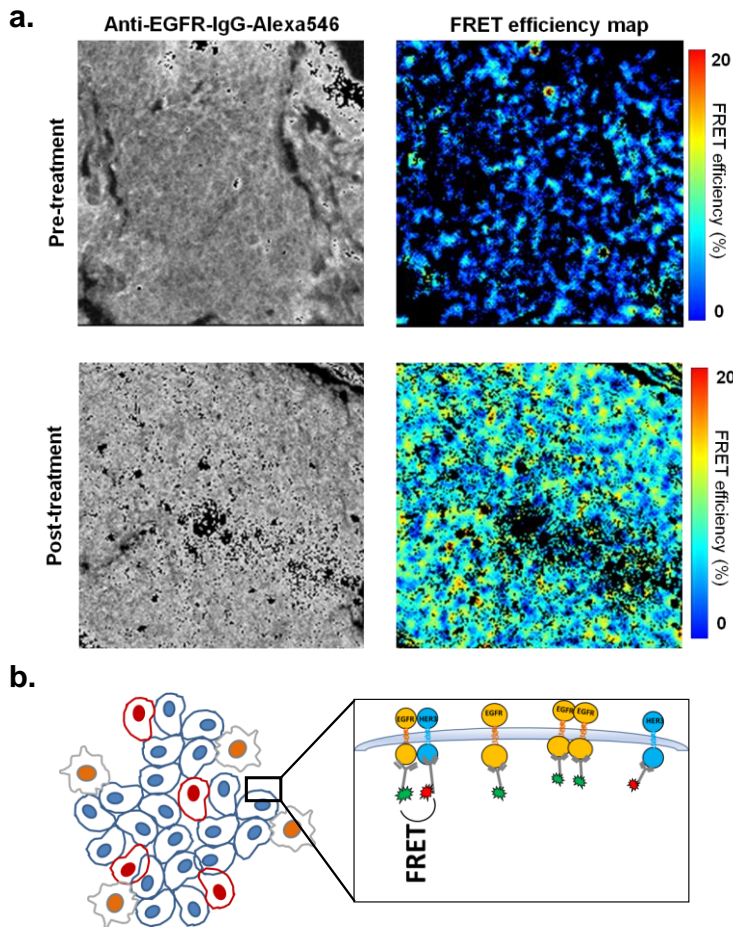


Figure 1. Increased HER1-HER3 interaction in breast cancer during neoadjuvant treatment with cetuximab (as previously described by Tao et al[1]) comparing pre-treatment biopsy and post-treatment surgical sample. **a.** Time –resolved intensity and FRET efficiency maps before and after the treatment. FRET efficiency was calculated $E_{\text{FRET}} = 1 - \tau_{\text{DA}}/\tau_{\text{D}}$ where τ_{DA} is the donor lifetime in the presence of acceptor and τ_{D} is the donor lifetime in the absence of acceptor. Receptors were fluorescently labelled with Anti-EGFR-IgG-Alexa 546 and Anti-HER3-IgG-Cy5 antibodies. **b.** Schematic illustration of the tumour tissue and receptors staining using the above mentioned intra-cellular antibodies. Carcinoma cells are shown in red and blue to visualize cellular heterogeneity. Macrophages are shown in grey.

seen by an increase in EGFR-HER3 dimer quantities with treatment.

Another example is the HER2-HER3 dimer which has been shown to drive proliferation and tumour progression. Targeting of this dimer with pertuzumab alongside chemotherapy and trastuzumab, has shown significant clinical utility. By quantifying the HER2-HER3 dimer in 131 formalin fixed paraffin embedded (FFPE) breast cancer tissue microarrays from METABRIC patients we found that the extent of HER2-HER3 dimer formation predicts the

after the treatment (high FRET efficiency shown as turquoise/orange in the donor lifetime pseudocolor map). We established that there is a physical interaction (rewiring) between the two receptors in resistance development, where EGFR-targeted therapies in TBNC patients residual cancer burden post neoadjuvant EGFR therapy, were shown to induce HER3 activation as well as an increase in HER3 in 70% of patients, pointing towards EGFR-HER3 rewiring post-treatment. We suggest that this change in receptor status may prevent therapy induced tumour regression. Two key points highlighted in this study were the importance to enrol patients on therapies specific to multiple receptor status (changes in the abundance of EGFR and HER3 in residual tumours), as well as evidence of oncogenic-rewiring in patients treated with anti-EGFR therapies that can be

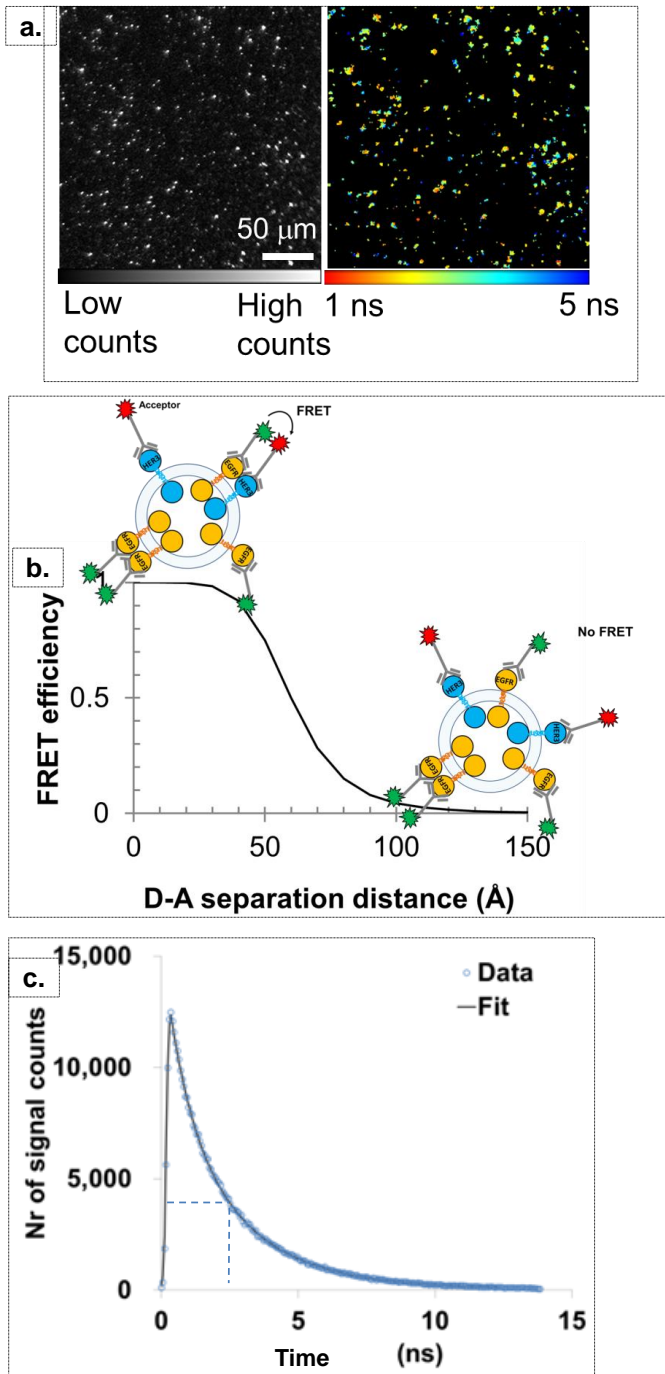


Figure 2. FRET/FLIM fluorescence assay of circulating exosomes extracted from H1975 lung cancer tumour xenografts mice serum. **a.** Time-resolved intensity images and donor lifetime map of exosomes labelled with Anti-EGFR-IgG-Alexa 546 and Anti-HER3-IgG-Cy5 extracellular antibodies. **b.** Schematic illustration of the fluorescent labelling geometry on exosomes and distance-dependence of FRET efficiency. **c.** Fluorescence decay data and exponential fit of the signal counts collected from all the pixels in the image shown in Fig. 2a. The fluorescence lifetime is at $1/e$ of the fluorescence intensity at time $t=0$.

likelihood of metastatic relapse up to 10 years after surgery, independently of HER2 expression[53].

This technique could be applied to assess various HER receptor dimerization and other protein interactions by varying the primary antibodies. Of further interest would be to explore the predictive value of EGFR/cMet dimers. Other methodologies of HER receptor quantification which can give positive results at a distance between substrates of 30-40nm such as ligation of proteins have been reviewed elsewhere [54]. However, FRET is used to assess protein interaction for distances less than 10 nm. FRET/FLIM methods are the most sensitive in their ability to determine small changes in interaction distance and thus likely to represent direct interaction between receptors.

Even though we have successfully used FRET/FLIM to quantify various heterodimers in human cancer tissues, the invasive nature of biopsies makes FLIM histology less

attractive to longitudinally monitor patient response to therapy. Molecular analyses similar to those in human tissues may be possible on a new type of liquid biopsy for solid tumours based on analysing exosomes in the patients' blood.

5. Circulating exosomes

Back in 2014, Ng and Beck proposed an alternative approach for personalised cancer medicine – the “C2c (cancer to chronic disease)” approach[55]. Their proposal was to convert current cancer treatments into regimens throughout the disease progression that mimic manageable chronic diseases in the clinic, by combining different state-of-the-art tools and strategies taken from multiple disciplines (physics, biology, and medicine). A key part of C2c was to develop advanced tools for liquid biopsy analysis. The advantages of liquid biopsy over tissue biopsy are obvious. Firstly, the minimally invasive procedure of obtaining patient blood will allow for frequent and longitudinal collection of samples, offering continual monitoring of the cancer throughout the disease progression. Secondly, collecting samples from circulating blood allows the capture of information originating from tumour sites all around the body. In contrast to the solid biopsy of a local region – which has long been known to be an inaccurate representation of the patient's cancer status [5, 56] – liquid biopsies could potentially overcome inter- and intratumour heterogeneity. Lastly, there is a plethora of information that can be obtained from a single blood sample. This includes circulating tumour cells, DNA, microRNA, proteins, onco-metabolites, and exosomes.

Exosomes are small (30 – 100 nm) vesicular bodies of endocytic origin, and are secreted by most cells in the body as a type of extracellular vesicles (EVs). They were first observed in 1981. In 1983 the secretion of these vesicles from multi-vesicular bodies (MVBs) were observed, then confirmed in 1985, and finally termed ‘exosomes’ in 1987[9, 11]. Tumours are also known to secrete EVs, including exosomes. However it must be noted that the amount and content of these EVs and exosomes varies between different types of cancers, therefore the concentration of tumour exosomes in any given amount of patient serum is not known[7, 11]. Exosomes are unique in the way that they affect other cells. Even before the term ‘exosome’ was coined, Poutsika et al had already demonstrated that melanoma MVBs are capable of activation of immune cells and interfere with immune response[57]. Wolfers et al demonstrated that exosomes can present tumour antigens to dendritic cells to activate anti-tumour response[11]. The immunogenic effects of tumour-derived exosomes were later demonstrated by others *in-vivo*[7, 8, 58]. *In vitro* studies have shown many pro-tumourigenic functions of these tumour derived exosomes, such as their interactions with local and distant microenvironments (such as the stroma) to increase cell motility, promote migration and metastasis[10, 59-63], as well as proangiogenic effects upon interactions with endothelial[64,

65]. All these are possible because of their specific biogenesis pathway, which allows the vesicular contents as well as receptors found on the vesicular surface to remain functionally active. By targeting these active RTKs on the surface of exosomes, Coban, Weitsman and Ng et al. (data not published) have recently demonstrated that it is possible to apply FRET/FLIM technology to interrogate the HER heterodimers on circulating exosomes from lung cancer xenografts (Fig. 2). Fig. 2a shows the time-resolved intensity image and the corresponding donor lifetime pseudocolored map of circulating exosomes. Exosomes were extracted from mice serum using an optimized repeated centrifugation protocol as previously published and imaged after simple adsorption to a glass surface [9]. The receptors of interest were labelled with extracellular Anti-EGFR-IgG-Alexa 546 and Anti-HER3-IgG-Cy5. Fig. 2b shows the dependence of FRET efficiency on the D-A separation and illustrates the high FRET efficiency detection when the receptors are dimerized and no FRET occurring when the donor and acceptor labelled molecules are not in close proximity. The distance dependence of the FRET efficiency is plotted for a (D,A) pair with a Foerster radius of 60 Å. The fluorescence decay data and exponential fit of the photon counts collected from the exosome imaged in Fig. 2a is shown in Fig. 2c. The fluorescence life-time is determined by the time point at which the fluorescence intensity drops to 1/e of its value at the beginning of the measurement.

Considering the small size of exosomes (<100 nm) we utilized extracellular labelled antibodies to rule out FRET detection via simple proximity of the receptors cytoplasmic domains within these small extracellular vesicles. Their size also limits their accurate detection and imaging by conventional microscopy which is limited by optical diffraction. However, current single-molecule localization techniques have achieved resolutions of 20 to 50 nm which open the possibility of imaging and tracking of cancer-derived exosomes. Recently, using a generalized single-molecule high-resolution imaging with photobleaching (gSHRIMP) method based on quantum dot blinking - which achieved a 15 nm lateral resolution - we were able to show that the measured number of EGFR receptors per super-resolved cluster together with the intra-cluster distances have predictive power for the phosphorylation of mitogen-activated protein kinase in a Hcc1943 breast cancer cell model[66]. Exosomes are enriched with a subset of specific proteins of the tetraspanin family (CD9, CD63 and CD81), members of the endosomal trafficking proteins (ESCRT-related proteins/Alix), heat-shock proteins, mRNA and miRNA and play an important role in cell-to-cell communication. In the future, the use of single-molecule localization microscopy to image exosomes would allow for the analysis of cancer metastasis-specific miRNA or protein distribution on the nanoscale which may provide new insights into the processes driving metastasis.

As pointed out by Beck and Ng[55], liquid biopsy is imperative for the C2c approach in the management of cancer. When serology-based *-omics* are used alongside imaging, the combined sensitivities and specificities of molecular imaging and next-generation sequencing

techniques will be able to facilitate early detection of the treatment-resistant variants that evolve as a mechanism of acquired resistance. Examples of other biomarkers are prostate-specific antigen as exemplar protein biomarker, and 2-hydroxyglutarate as an example of epigenetic oncometabolite. By tracking liquid biopsies, clinicians will be able to monitor the ever-evolving *-omics* heterogeneity of the cancer, which in turn informs the next best treatment(s) as soon as any tumour evolution occurs. The ability to measure receptor status in circulating exosomes allows for the continual monitoring of cancer evolution in the long term. This is something that cannot be done by non-invasive imaging or biopsy, and thus makes a potentially valuable and novel tool that adds to the C2c approach.

6. Conclusions

It had long been observed that different cancers utilise different mechanisms to rewire their signalling cascades, which leads to resistance development against cancer therapies [35]. In order to improve current prognosis, outcome prediction, and long term efficacy of treatments in the clinic, it is important to gain an in-depth understanding of the underlying mechanisms of signalling rewiring that regulate drug resistance.

The initial exosomal measurement of receptor status at diagnosis will help making decisions on patient treatment, as an added parameter to other biomarkers [53]. The ability to measure receptor status in circulating exosomes also allows the continual monitoring of cancer evolution in the long term. Upon identification of oncogenic rewiring, via FRET/FLIM observation in changes of receptor dimerization status, clinics can use this information to adjust the treatment used for each individual patient. Strategies include designing combinational and/or adjuvant therapies to suppress the newly rewired signally cascades, or in some cases even changing the whole regimen altogether to counteract any observed oncogenic rewiring in the individual. As suggested by many and demonstrated by some studies, this combinatorial targeted approaches have very positive outcome in suppression of the rewired resistances[16, 33, 35, 53, 67, 68].This longitudinal monitoring of receptor dimerisation sheds light on the potential of C2c approach to personalized medicine. Taken together, FLIM histology and exosomal FLIM can be combined to form a powerful tool for monitoring receptor signal rewiring to predict clinical outcome as well as to stratify treatment. The ultimate objective is to use protein network rewiring information to stratify and guide future therapies that can overcome primary or acquired resistance and either turn cancer into a chronic disease or achieve cure.

With rising interest in exosomes as a platform, receptor dimers as a biomarker, and exosomal FRET/FLIM as a potential tool, many ongoing studies and clinical trials in different cancers - including colorectal[69], breast[70], lung[71], head and neck [72], and more - are all producing

data that are compiling and contributing to bringing different aspects of this into clinic. Whilst still a long way from completion, the C2c approach of combining biology, physics and medicine to stratify and personalise cancer medicine will take a major step forward with the addition of this new tool.

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