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Using Clinical Proteomics to Discover Novel Anti-Cancer Targets for MAb Therapeutics

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1. Introduction

1.1 A brief history of basic cancer research driven target discovery

The incidence of cancer has risen dramatically over the last century due to the improved treatments of chronic diseases and as a consequence of an ever aging population that results in elevated cancer development(Hanahan and Weinberg 2000). As a result of the dramatic rise in cancer incidence significant efforts have been placed on developing new strategies for the improved diagnosis and the treatment of cancer. Traditionally, ionizing radiation and/or cytotoxic chemotherapies like platinum derivatives and anti-metabolites have been most widely used as anti-cancer treatments and although these agents can still have significant effects on improving longevity in patients, the toxicity of these agents along with high frequency of acquired resistance requires the development of more specific anti-cancer agents.

International research efforts over the past thirty years have revolutionized our understanding of cancer developments. Pioneering distillation of this knowledge has led to the development of "the hallmarks of cancer" that specifies specific pathway perturbations in the development of the disease(Hanahan and Weinberg 2000). These rate-limiting stages are comprised of numerous biological systems that need to be perturbed during the multi-step evolution of the full cancer cell. The key systems maintaining tissue integrity and which are perturbed in cancer development include pathways that mediate genome instability, inflammation, sustained cell proliferation, evasion of tumour suppressor signals, death resistance, immortality, angiogenesis, metastasis, immune system silencing, and energy metabolism (Table 1).

This research knowledge summarized above has been acquired using a range of approaches including the study of cancer causing animal viruses like SV40 or human oncogenic viruses like HPV, the use of "model" organisms like bacteria, yeast, worms, and flies that recapitulate apoptosis and cell cycle concepts, and the development of human cancer cell lines that mimic certain aspects of cancers. Despite this enormous knowledge of cancer mechanisms, there have been relatively few anti-cancer drugs developed for routine clinical use from such information. In fact, the most "targetted" and common anti-cancer agents are those that either exploit knowledge of the physiology of cancer (like the anti-estrogen Tamoxifen) (Easton, Pooley et al. 2007) or those that exploit the use of natural products like

Cancer Hallmarks	
I. Sustained Proliferation	growth factor ligands activate kinase growth signalling cascades growth factor independence via kinase activation or gene mutation genes include MAPK, B-RAF, AKT Therapeutic Mabs target tyrosine kinases
II. Evasion of Tumour Suppressors	mutation or inactivation of tumour suppressor proteins genes include p53, RB, NF2 Therapeutic Mabs target receptors elevated in p53$^-$ cells like CD44
III. Inhibition of Cell Death Signalling	induction of anti-apoptotic programmes suppression of pro-apoptotic programmes genes includes bcl-2, bax, beclin
IV. Immortality	suppression of normal senescence programmes gene include Telomerase
V. Angiogenesis	reprogramming of tissue vascularization genes include vEGF, FGF, Thrombospondin Complex cell models required for further understanding Therapeutic Mabs target FGF receptor family
VI. Metastasis	reprogamming of cellular mechanisms of adhesion and migration genes incude E-Cadherin, EMT pathway (SNAIL, SLUG, TWIST) Complex cell models required for further understanding Therapeutic Mabs target integrins
VII. Immune System Evasion	Cellular resistance to cytotoxic T-cells and NK-cells genes include IRF-1, chemokine ligands, interluekins Complex animal models required for further understanding Therapeutic Mabs target T-cell receptors and CXC-receptors
VIII. Energy Metabolism	Reprogramming of cells to aerobic glycolysis Adaption of cells to hypoxia genes include GLUT1/3, HIF-1, TCA cycle gene mutation, CA-IX Therapeutic MAbs target Glucose transporters and CA-IX

Table 1.

Taxol (that targets microtubules and effects cell division)(Cowden and Paterson 1997). This is not to say that there are no novel agents being developed from our vast knowledge of cancer mechanisms; a large panel of small molecules have indeed been developed that target specific protein kinases, acetyltransferases, proteases, protein-folding enzymes, and other pro-oncogenic enzymes. Some of these small molecules form at the least proof-of-concepts in cancer models for the drug-ability of classes of enzyme: (i) cell cycle enzymes such as CDK1/2 are targeted by a small molecule named Roscovitine(Meijer, Borgne et al. 1997; Kim, Alarcon et al. 2009; Rule 2011); (ii) the protein folding enzyme HSP90 is targeted by the anti-tumour anti-biotic Geldanamycin (Kim, Alarcon et al. 2009); (iii) the protease component of the proteasome is targetted by a small peptide-mimetic Velcade (Rule 2011); (iv) a pro-apoptotic protein-protein interaction is mimicked by the tetra-peptide mimetic SMAC(Wang, Lu et al. 2011); (v) the E3 ubiquitin ligase MDM2 is targetted by a peptide mimetic named Nutlin(Vassilev, Vu et al. 2004); and (vi) histone de-acetylases are targeted by small molecules named Sirtuins(Medda, Russell et al. 2009). Although the majority of such drug-leads are not in the clinic, many of these pro-oncogenic enzymes will likely form important targeted therapies in the future. However, there is also some thought that the small molecule "landscape" that can be exploited to develop specific anti-cancer drugs has been relatively exhausted and that the new frontier involving "biologics" will provide a more revolutionary approach for treating large populations in the long-term.

2. The future of cancer therapeutics using clinical cancer models and biologics

In addition to the classic drug discovery process using small molecules that target enzyme pockets, the development of biologics, including the use of peptides, monoclonal antibodies, and aptamers, which has lagged the development of small anti-cancer molecules based on classic chemistry, will likely form a growing niche in the future. In part, this is due to the fact that the pharmaceutical industry has had one hundred years to develop the expertise of organic chemistry and has in a sense saturated the small molecule landscape. The exploitation of monoclonal antibodies that target extracellular receptors and/or peptide-aptamers that target the more difficult protein-protein interaction is only approximately 20

years old. However, the use of biologics like monoclonal antibodies and to a lesser extent protein-protein interaction inhibitors is emerging as a major market leader in all cancer based therapeutics. There are currently 10 FDA approved monoclonal antibodies for the treatment of cancer (Table 2).

Antibody	Brand name	Approval	Type	Target	Indication
Rituximab	Rituxan	1997	Chimeric	CD20	NHL
Trasuzumab	Herceptin	1998	Humanized	ErbB2	Breast Cancer
Gemtuzumab	Mylotarg	2000	Humanized	CD33	AML
Alemtuzumab	Campath	2001	Humanized	CD52	CLL
Ibritumomab tiuxetan	Zevalin	2002	Murine	CD20	NHL
Tositumomab	Bexxar	2003	Murine	CD20	NHL
Bevacizumab	Avastin	2004	Humanized	VEGF	Colorectal cancer
Cetuximab	Erbitux	2004	Chimeric	EGFR	Colorectal cancer, Head and Neck cancer
Panitumumab	Vectibix	2006	Human	EGFR	Colorectal cancer
Ofatumumab	Arzerra	2009	Human	CD20	CLL, NHL, DLBCL

Table 2. FDA approved mAbs for cancer treatment.

One of the largest sectors in the current pharmaceutical market is Oncology, which is largely due to the lack of targeted drugs for improved treatment of the vast number of different cancer types. Traditionally anti-cancer drugs fall into two loose categories either those which are cytotoxic (platinum based drugs) or those, which go after specific targets/proteins affecting a particular pathway. While traditionally the cytotoxic based chemotherapies have been most widely used there is now a need for the more targeted approach. Of these targeted therapies monoclonal antibodies (mAbs) are emerging as the market leaders in all cancer based therapeutics holding an 80% share of the US targeted cancer market (Aggarwal 2010) . Monoclonal based therapeutics have been around for the last 20 years with 30 immunoglobulins (IgGs) approved for a variety of conditions. One of the major limitations of monoclonal antibody therapeutics is target selection. While many companies first selected the clinically validated targets in the literature this area has become over crowded. Companies are now developing 2nd and 3rd generation antibodies towards these same targets either towards different epitopes, longer half-lives or with different scaffolds. (Beck, Wurch et al. 2010). However, the academic research and medical community has a key role to play in the target discovery process. This will greatly increase the pool of targets with which the pharmaceutical industry can exploit to begin to improve the costly process of developing novel anti-cancer treatments.

2.1 Targeting oncogenic kinase receptors using mAbs

Despite the fact that there are currently a paucity of well-defined, clinically accepted targets in oncology, a good example of a monoclonal antibody arising from physiologically relevant cancer model comes from the history of the development of the monoclonal that targets Her2, tratsuzumab (herceptin). Her2 was discovered as an ocogene activated by point mutations in chemically induced rat neroblastomas (Schechter, Stern et al. 1984). HER2 is a

type 1 transmembrane glycoprotein divided into three domains: an N-terminal extracellular domain (ECD), a single α-helix transmembrane domain (TM), and an intracellular tyrosine kinase domain. The extracellular domain forms a large structure capable of binding a ligand although no known ligand has yet been found for Her2. Although no ligand is known for Her2 it is known that its dimerization with the other Her family members is required for its activation by inducing auto-phosphorylation of its intracellular tyrosine kinase domain. From its inception in the early 80's in rat neuroblastomas Her2 later went on to be shown to be overexpressed in a subset of breast cancers, with over-expression leading to poor prognosis (King, Kraus et al. 1985; Slamon, Clark et al. 1987). It was this association with overexpression and poor prognosis that led to the development of antibodies towards the extra-cellualar regions of these receptor tyrosine kinases. The resulting antibody named tratsuzumab was a humanized mouse monoclonal antibody and was later approved by the FDA in 1998 for treatment of metastatic breast cancer (Slamon, Leyland-Jones et al. 2001).

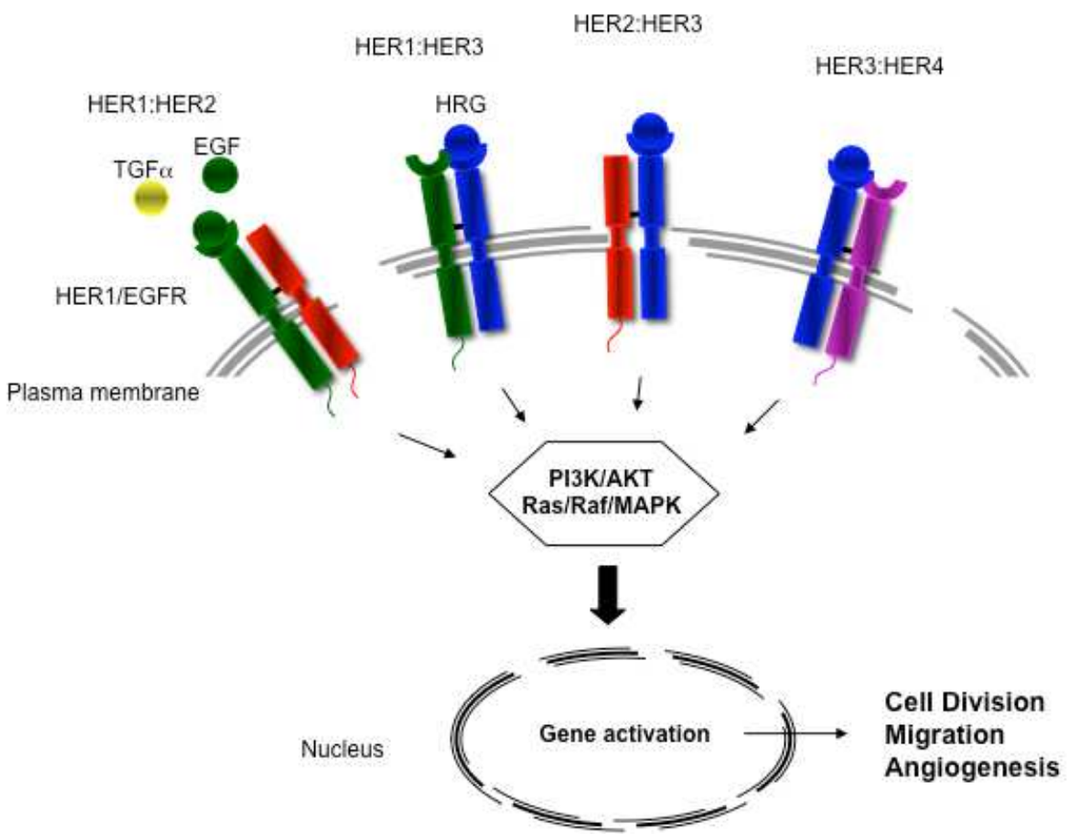

Fig. 1. **The Her2 Signalling pathway**. HER signaling pathway in breast cancer. There are four membrane receptor tyrosine kinases involved in Her signaling (HER1[EGFR],HER2, HER3 and HER4) and a number of known ligands (epidermal growth factor [EGF],transforming growth factor a [TGFa] and heregulins [HRG]). Ligand binding induces conformational changes in the receptor allowing homo and hetero-dimerization transmitting signals via phosphorylation on intra-cellular domain activating PI3K/AKT, RAS/MEK/MAPK cascades. The monoclonal antibody trastuzumab targets the HER2 receptor preventing it from dimerizing and activating its singaling pathways.

Tratsuzumab binds to a site close to the transmembrane region which is out with the potential ligand docking site effecting dimerization with other Her family members (Tai, Mahato et al. 2010). It was an instant success when used along side conventional chemotherapeutic agents with recurrence rates halved and mortality dropping by 30% which was seen to far out weigh the small risk of cardiotoxicity sometimes seen (Chang 2007). Such data highlight the utility of identifying receptors as anti-cancer targets and mAbs in particular as tools with which to develop specific and effective anti-cancer agents. However, this history highlights the time scale involved in the process from target discovery to clinically approved use of an agent.

2.2 Targeting hypoxic cells using mAbs

Another example of a physiologically relevant cancer target that is proving to be important in oncology is Carbonic anhydrase-IX (Thiry, Dogne et al. 2006). One of the key hallmarks of cancers is the change in metabolism linked in part to lowered oxygen supply (Hanahan and Weinberg 2011). In human cancers, hypoxia develops from an inadequate supply of oxygen, which is primarily a physiological change in the tissue due to structurally disturbed microcirculation and reduction in O_2 diffusion. Solid tumor growth is limited by vascularization and indeed promoted by angiogenesis, which is necessary for oxygen and nutrient supply. Hypoxia can induce the expression of many genes, including the key transcription factor HIF-1 that can in turn facilitate the induction of genes implicated in altered metabolism such as GLUT1/3 glucose importers and Carbonic Anhydrase IX (Figure 2).

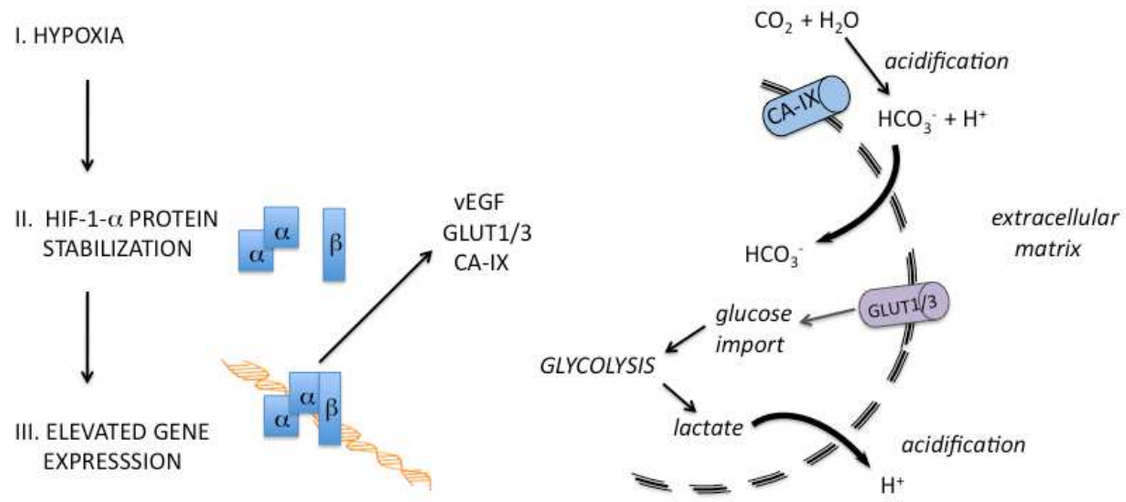

Fig. 2. **Regulation of extracellular acidification and glycolysis in hypoxic cells**. The transcription factor HIF-1a is degraded in normoxic cells through the VHL tumour suppressor protein. In response to lowered oxygen concentrations, HIF-1a is stabilized and forms a complex with HIF-lb to induce adaptive gene products. Some of these gene products include CA-IX and GLUT1/3 that coordinately mediate glycolysis and acidification that together drive survival in cancer cells. GLUT1/3 drives glucose transport where cellular energy is derived from glycolysis. CA-IX catalyzes extracellularly condensation of carbon dioxide and water to hydrogen ions and carbonate. Carbonate ions are transported intracellularly where CA-II neutralizes hydrogen ions and carbonate to carbon dioxide, which is transported from the cell.

The latter enzyme surprisingly functions as an intrinsic marker of cancer hypoxia and numerous mAbs and small molecule leads have been developed that target and exploit the hypoxia-specific nature of this isozyme. Many potent CA inhibitors derived from acetazolamide, ethoxzolamide and benzenesulfonamides have been shown to inhibit the growth of several tumor cell lines in vitro and in vivo(Vullo, Innocenti et al. 2005), although the specificity for isoforms and other enzymes like Aquaporin might add to the therapeutic responses. mAbs have also been developed independently to CA-IX and numerous studies have been developed showing that such mAbs can be used to target in vivo hypoxic regions of cancers (Zatovicova, Jelenska et al. 2010). Such mAbs might be used therapeutically as inhibitors of the enzyme or more possibly as carriers of cargo in a focussed attempt to increase local concentration of cyto-toxics.

2.3 Exploiting the immune system using mAbs

Immunological disorders have successfully been treated by targeting cytokines and associated receptors with a number of mAbs currently approved and many in clinical trials. A major imitation of most animal cancer models such as xenografts in immune compromised nude mice, not to mention the use of invertebrate model organisms or human cancer cell lines, is that the immune system is not taken into account as a variable factor. With so much known about the pathways involved in immunological conditions and so many mAbs in development and their success rate relatively high there is little need to identify new targets in this area. Cancer on the other hand is a different story due to its ever-evolving state. Cancer arises from multiple mutations driving the proliferation and survival of the cell so to put the brakes on this requires hitting the right targets (Weiner, Surana et al. 2010). Going for the validated targets provides a route from which companies can get to market quickly with their blockbuster drugs but this is getting increasingly harder. Until now many have steered away from discovering totally novel targets as this is more risky and the road to market is much longer, but the proteomic technology is now available and reliable to analyze clinical tissue and identify novel cancer targets. (Beck, Wurch et al. 2010)
To date most targeted cancer therapies which have entered the clinic have done so by being used in combination with other cytotoxic agents. A good example of this is bevacizumab an anti-VEGF antibody, which alone showed no survival benefit but used in combination with oxaliplatin and 5-fluorouracil provided a two and a half month survival advantage in metastatic colon cancer (Kerbel 2006) . Cancer treatment is made even harder by the ability of cancer cells to overcome the toxicity of drugs by becoming resistant to the drug themselves. This ability has led many to people proposing the only way to treat cancer is via a drug combination strategy (Sawyers 2007). To achieve this goal we need to discover novel targets and develop new drugs which can be used in synergy or as second, third line treatments to combat the ever evolving mechanisms of cancer to evade treatment.

2.4 BiTE therapy

Bi-specific antibodies are also being explored in the treatment of some malignancies with the aim to engage the patients' immune system to attack the tumor. The emerging leader in this field is blinataumomab, an anti CD19 and CD3 bi-specific single chain BiTE antibody (Bargou, Leo et al. 2008). With the knowledge that T cells are involved in the immune surveillance of cancer and their presence within tumors can effect the patients outcome, it is not surprising that exploiting T cells to control tumor growth is being explored (Swann and

Smyth 2007). Over the last 20 years many attempts have been made to achieve this such as vaccines, ex-vivo expanded T cells and T cell-activating antibodies, either alone or in combination with treatments such as IL2, IFN2a and GM-CSF to stimulate T cells (Nagorsen and Baeuerle 2011). These attempts thus far have yielded relatively low response rates presumably due to the nature of cancer to escape the pressure put on them by these treatments by losing molecules involved in T cell recognition for example.
Bi-specific antibodies can overcome the ability of cancer to evade such treatments by passing the need for a cancer cell to posses the ability to recognize T cells themselves. One arm of the the bi-specific antibody is targeted towards a surface antigen on cancer cells such as EGFR, Her2, or cell differentiation antigens such as CD20, CD22 or CD19. The second arm of the antibody is then targeted towards an activating component of the T cell such as CD3. The binding of both of these antigens brings together the cancer cells and T cells resulting in the activation of any cytotoxic T cells causing lysis of the tumor cells. The BiTE antibody blinatumomab is currently in clinical trials for use in NHL and ALL with promising results so far (Nagorsen and Baeuerle 2011).

3. Novel cancer target discovery using proteomic approaches

It is important to point out that many of the targets of monoclonal antibodies like those reviewed above were not discovered from basic cancer research using model organisms like yeast, worms, and flies, but were discovered from understanding using human cancer material the extracellular proteins driving cancer growth or resistance and exploiting knowledge of the immune system as a co-factor in cancer control. The development of "OMICS" platforms like transcriptomics and proteomics over the past ten years and the application of these OMICS approaches to human cancer tissue has revolutionized target discovery in the cancer field and has identified a large panel of novel anti-cancer targets that had not been previously realized. This highlights the need to develop approaches that can be used to interrogate clinical samples and place less emphasis on the use of model organisms like yeast, bacteria, and flies as doors to discover novel cancer targets. In fact, not only do such model organisms fail to model the "hallmarks" of cancer like immunity and metastasis, they do not express many of the oncogenic targets that are proving to be important in human cancer. This in part is due to the gene explosion in the animal lineage that accompanies complex vertebrate life; such advanced life is linked to complex problems faced by vertebrates that include multi-functional organs as well as much longer life spans and an immune system to combat pathogens that could easily colonize the rich tissue niches. It is also due to the surprising loss of some cancer genes on the evolutionary path of flies and worms. For example, the p53 tumour suppressor and its co-factor MDM2 appeared together very early in animal evolution and it was surprising to learn that the model organisms like flies and worms have lost these 2 key genes(Lane, Cheok et al. 2010). Thus, although an advantage of using invertebrate model organisms is that genetic screens coupled to rapid life span can be used to identify growth regulatory targets, such organisms do not have many of the oncogenic targets that are important for human cancer. An example of vertebrate-specific cancer gene discovered since the OMICS revolution was a gene named Anterior-Gradient-2. This gene and its orthologue AGR3 evolved from a founding gene that is in invertebrates named ERP18. The AGR2/AGR3/ERP18 family has since been under strict evolutionary control with no subsequent gene amplification and diversification, suggesting that the function of this family of vertebrate-specific genes is highly integrated and fixed. AGR2 specifically (i.e. not AGR3 not ERP18) plays a fundamental role in limb

regeneration in amphibia, acts like a classic transforming oncogene, can mediate metastasis in animal models, and its expression can predict poor prognosis in various cancer types(Hrstka, Nenutil et al. 2010). Another important cancer gene that functions not only as a tumour suppressor but plays a key role in extending life span in mice, is a gene named ARF (p14 or p19 in human and mouse, respectively)(Maclaine and Hupp 2009). This gene interestingly is only in mammalian lineage and is one of the fastest adaptively evolving genes in mammals suggesting a key role in host-pathogen arms race that typifies genes under positive evolutionary pressure. There are other such cancer genes that are not present in classic invertebrate model organisms and highlights further the need for better clinical models to accurately understand the physiology of human cancer.

The limitation on the use of clinical tissue to identify novel and/or potential anti-cancer protein targets stems form the paucity of well-characterized and well collected clinical samples. That is to say, the methods for protein identification in clinical tissue are adequate, but the development of useful clinical models lag the technological developments. Traditionally or originally, examinations of differentially expressed proteins have utilized "tumour" vs "normal" tissue and exploited differential protein expression methods to identify proteins "over-expressed" in tumour vs normal tissue. Such protein differential display techniques (now called proteomics) including 2-d gel electrophoresis linked to mass-spectrometric fingerprinting has been available for almost twenty years. However, only with the full sequencing of human genome, software to annotate all potential open reading frames, and the use of MS/MS to sequence directly peptides and match peptides to the human proteome was relatively large scale proteomics screening useful. There are now many examples where differential protein expression can be quantitated in clinical tissue using mass spectrometric methods including iTRAQ(Collins, Lau et al. 2010) or label-free and data-independent methods like PAcIFIC (Panchaud, Scherl et al. 2009). Historically, most MS/MS protein sequencing has been performed using reversed-phase liquid chromatography (HPLC) tandem mass spectrometry (MS/MS) approaches with data-dependent ion selection. This so-called shotgun proteomics is combined with liquid or gas phase fractionation to increase coverage of the proteome, which is relatively speaking, still limiting. The recently described PAcIFIC has two advantages over these powerful, but traditional data-dependent shotgun proteomics approaches. First, because every *m/z* channel is evaluated systematically to collision induced dissociation (CID) there is an increase in the dynamic range of proteins identified. This permits detection of a larger number of low abundant proteins, such as kinases and transcription factors, that may have relatively weak precursor ions available for detection. Second, because of the thorough nature of PAcIFIC, protein sequence coverage is dramatically improved which increases confidence in protein identification, that is another contentious topic in the proteomics fields. Another generic limitation of full proteome coverage is in part due to biological issues like the fact that buffers used for lysis cannot extract all cellular proteins. Thus cutting edge proteomics approaches using mass spectrometry aim to elevate the coverage of proteins sequenced in a biological sample.

In addition to the strength and limitations of protein sequencing methods from biological tissue, it is also becoming apparent that examination of proteins differentially expressed in cancer versus normal tissue is not specifying adequate target-discovery. Many studies have compared cancer vs normal tissue in discovering differentially expressed proteins, but these data sets do not identify specific drivers of the disease because the tissues are very different from each other. More thoughtful approaches and study designs are required to identify higher-confidence anti-cancer drug targets that involve progression models where disease

zones have evolved from precursor tissue regions. Some examples of such clinical proteomics approaches will be described below.

3.1 Discovering novel p53 regulators using clinical proteomics

Cancer development is a multi-step process which involves clonal evolution of cells under natural selection resulting in the survival of cells with an enhanced capacity to evade normal growth control (Rajagopalan, Bardelli et al. 2002). The core genetic blueprint of the cancer cell is being developed and involves universal perturbation in sets of signal transduction pathways regulated by well-studied proteins such as RAS, p16, and p53 (Hanahan and Weinberg 2000). Of these many pathways, one of the most frequently mutated and silenced genes in human cancers is p53 (Vousden and Lane 2007). The tumour suppressor protein p53 is a stress-activated transcription factor that mediates cellular response to a diverse range of environmental signals including DNA damage, virus infection, and metabolic stress (Levine, Hu et al. 2006) (Hupp and Walkinshaw 2007). The most widely studied p53 inhibitor, the MDM2 oncogene, is often over-produced in a range of cancers resulting in attenuation of the p53 response (Levine, Hu et al. 2006) (Arva, Gopen et al. 2005).

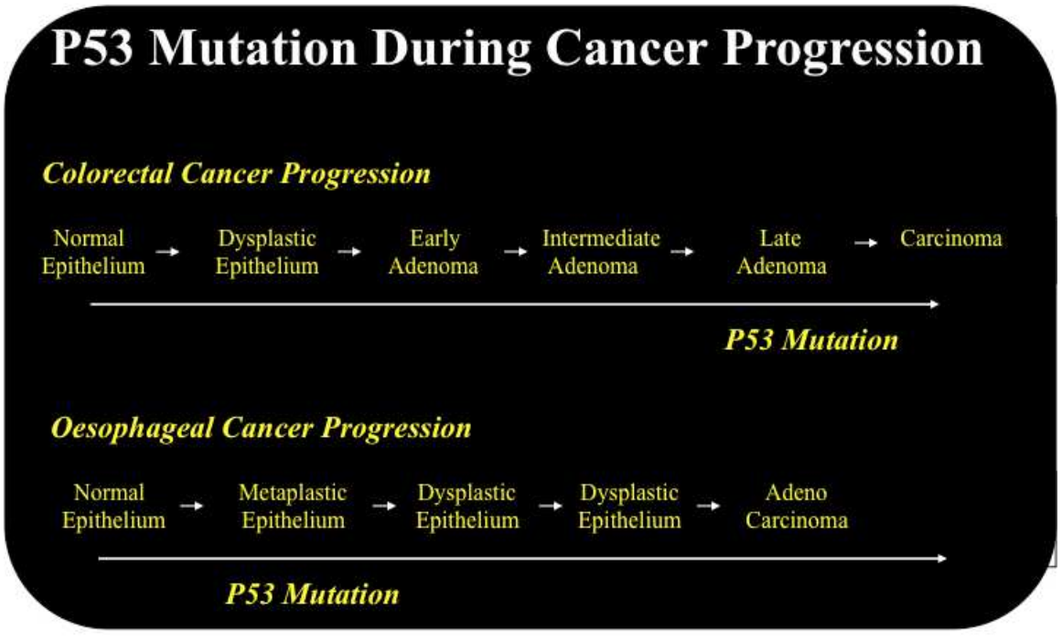

Fig. 3. **Model describing stage and tissue-specific mutation of the p53 gene in cancer progression.** Tissue morphology changes in the so-called cancer progression sequence has been categorized as metaplasia, dysplasia, adenoma, carcinoma, and metastasis. Key gene mutations occur at different stages in colorectal cancer and oesophageal cancer; colorectal cancer develops through mutations of APC in dysplasia, RAS in adenoma, and p53 in carcinoma. By contrast, oesophageal adenocarcinoma, that is linked in part to the DNA-damaging effects of acid and bile reflux, progresses through p16 and p53 mutation in metaplasia and through to RAS mutation in carcinoma. The unusual feature of oesophageal cancer is the very early selection of the p53 gene mutation very early in "progression" and highlights a novel opportunity to understand the environment-gene-proteome interactions in disease.

However, there is a need to develop novel p53 inhibitory screens in vertebrates, if not in human cancers, in order to expand our knowledge of cancer-causing gene pathways and to identify the proteome of novel and uncharacterized targets.

One approach to discover novel clinically relevant oncogenes that inhibit p53 would be to analyze key clinical tissue for gene products implicated in p53 silencing in human diseases using OMICs technologies; i.e. proteomics or transcriptomic methodologies. In order to identify potentially novel p53 inhibitors in clinical tissue, a clinical proteomics screen had previously been set up in a proliferative disease (Barrett's epithelium) which is an intermediate in the development of oesophageal adenocarcinoma. The notable feature of Barrett's epithelium is that selection pressures including acid and bile stresses are being placed on mutation of the p53 gene(Jankowski, Provenzale et al. 2002; Leedham, Preston et al. 2008) suggesting a relatively unique microenvironment for the identification of novel p53 modifiers (Hupp 2000; Darragh, Hunter et al. 2006; Little, Nelson et al. 2007). It is under this unique microenvironment that potentially novel pro-oncogenes might be identified. Accordingly, the proteomics approach to identifying a range of proteins upregulated in this proliferative tissue using clinically derived material identified a protein named Anterior Gradient-2 (AGR2) which was validated as a potent inhibitor of p53-dependent transcription(Yagui-Beltran, Craig et al. 2001; Pohler, Craig et al. 2004). AGR2 was originally identified as a potential secretory protein that is highly expressed in Xenopus eggs (Aberger, Weidinger et al. 1998). Apart from its function as a p53 inhibitor (Pohler, Craig et al. 2004), subsequent studies have shown a significant role for AGR2 in a range of biological pathways including cell migration, cellular transformation, metastasis (Liu, Rudland et al. 2005) (Wang, Hao et al. 2008), and limb regeneration in vertebrates (Kumar, Godwin et al. 2007). Clinical studies have also implicated the protein in inflammatory bowel disease (Zheng, Rosenstiel et al. 2006), hormone-dependent breast cancers (Zweitzig, Smirnov et al. 2007) (Mackay, Urruticoechea et al. 2007), and in predicting poor prognosis in prostate cancers (Zhang, Forootan et al. 2007). The molecular mechanisms underlying these wide-ranging biological pathways triggered by AGR2 are still not defined and as the AGR2 gene is confined to vertebrates, the AGR2 gene pathway cannot be analyzed and solved directly in genetic systems like yeast, flies, or worms. However, it remains important to determine what the interacting proteins-or Interactome-of AGR2 is in order to understand its role in development and cancer. One of the published binding proteins for AGR2 is the extracellular receptor linked to metastasis, C4.4A, possibly providing a therapeutic target for mAbs validation in AGR2-positive cancers.

3.2 Discovering novel Tamoxifen agonists using proteomics

Tamoxifen is one of the first generation smart therapies that can give rise to substantial improvements in disease management. However, tamoxifen has agonistic effects involving either intrinsic resistance or acquired resistance. This would indicate that there are factors differentially expressed in patients that confer tamoxifen resistance or that there are differences in tamoxifen induced proteins in patients that confer cancer cell growth. A number of transcriptomics approaches have been used to identify genes that are induced or suppressed by Tamoxifen that identify key rate-limiting stages of pro-oncogenicity. One of the most striking observations in Tamoxifen resistance is the suppression of the immune system regulatory factor IRF-1 and highlights again the important but under-studied link between suppression of the immune system and cancer development. However tantalizing the later data might be, this work was performed using cell lines and there is very little

evaluation of Tamoxifen agonist targets using clinical tissue in conjunction with proteomics. The identification of proteins that are induced by Tamoxifen would identify potential targets for sensitization strategies. A very recent proteomics study was designed using clinical samples to search for differentially expressed proteins in tamoxifen resistant or tamoxifen-sensitive patients and identified a number of proteins that when expressed predict poor response, thus highlighting the power of proteome screens using well-defined clinical cancer samples to identify effector proteins of the disease (Umar, Kang et al. 2009).

3.3 Discovering clinically relevant pro-metastatic targets using proteomics

One of the key problems in cancer responses to treatment is not necessarily the initial response, but the relapsed or metastatic development. Thus, one approach to improve cancer treatments would be to not target an oncogene that is over-produced in cancers, but to target the proteins that have been specifically upregulated in the relapsed or invasive cancers. Although this is relatively difficult in all cancer types, it is possible in breast cancers where biopsies can be taken from original and invasive lymph node samples. The development of such clinical proteomics screens was recently published by Bouchal, Roumeliotis et al. 2009 iTRAQ-2DLC-MS/MS proteomic analysis has been used to identify proteins overproduced in metastatic tissue vs relapsed primary tissue from the same patient. This study revealed potential new biomarkers and therapeutic targets in metastatic breast cancer tissue that would not be possible to predict form other research approaches. From this screen one of the most up-regulated trans-membrane proteins was identified as IFITM1.

Interferon-induced transmembrane protein 1 (IFITM1), also known as 9–27, CD225, and Leu13, is a member of the interferon-induced transmembrane protein family. Its major role is as a component of a membrane complex that transduces antiproliferative and homotypic adhesion signals in lymphocytes (Lewin, Reid et al. 1991; Sato, Miller et al. 1997). The CD19 cell surface receptor complex regulates B lymphocyte development and function and is composed of at least four proteins; CD19, CD21 (CR2, complement receptor 2), CD81 (TSPAN-28) and IFITM1 (Sato, Miller et al. 1997). Aside from its physiological role it has been shown to promote cancer progression by enhancing cell migration and invasion in head and neck cancer and gastric cancer (Yang, Lee et al. 2005; Hatano, Kudo et al. 2008). IFITM1 knockdown has also been shown to inhibit proliferation, migration and invasion of glioma cells (Yu, Ng et al. 2010). Therapeutic monoclonal antibodies have been developed targeting the CD19 protein and have show efficacy in chronic lymphocyte leukemia (CLL) and related CD19(+) B-cell malignancies (Awan, Lapalombella et al. 2009). Since IFITM1 has been shown to form a receptor complex with CD19, targeting it with monoclonal antibodies could also prove beneficial.

3.4 Novel mAb therapeutics in cancer

Another example of a more sophisticated approaches using a combination of proteomics for target discovery in cancer tissue to novel mAb therapeutics stems from the pioneering work of Dario Neri's laboratory. Conventional chemotherapeutics work by targeting highly proliferating tissues such as cancer but also target those normal tissues which also proliferate such as endothelial cells. The idea that antibodies can target cancer tissues alone relies on identifying a target that is only expressed in the tumor or at very least at a threshold that wouldn't have massive side effects in normal tissue. A popular approach to identify such targets is to evaluate the differential expression of proteins in cancerous vs normal tissues but finding the appropriate technique which will give the most accurate

results is difficult. Once method developed by Dario Neri is the biotinylation of proteins, this involves the in vivo perfusion of tumor baring animals or ex vivo perfusion of surgically resected human organs with reactive ester derivatives of biotin. The resulting biotinylated proteins can then be easily recovered using streptavidin followed by on-resin proteolyitic digestion and HPLC and finally mass spectrometry to identify proteins assessable in the circulatory system. This technique led to the identification of BST-2 as a novel target for antibody-based therapy (Schliemann, Roesli et al. 2010). Numerous proteomic techniques have been used over the years to discover new targets such as a phage display protocol where by mice are injected with a phage library and tissues excised and phage bound are identified (Pasqualini and Ruoslahti 1996). Direct analysis of tumor vs. non tumor vasculature via perfusion with colloidal silica beads (Jacobson, Schnitzer et al. 1992; Durr, Yu et al. 2004; Oh, Li et al. 2004). Finally a method of biotinylating proteins on the surface of endothelial cells or in the vasculature extra cellular matrix and subsequent proteomic analysis (Rybak, Ettorre et al. 2005; Roesli, Neri et al. 2006). Since tumors are continually proliferating they have a high demand for oxygen so require a very extensive vasculature system to supply their need. Targeting the tumor vasculature is an attractive approach to treat cancer and this technique has led to the identification of numerous potential targets present in tumor vasculature over normal vasculature.

Targeting the tumor neo-vasculature has been demonstrated by developing antibodies towards the EDA domain of fibronectin. This alternatively spliced extra-domain B (EDB) of fibronectin is one of the best characterized markers of angiogenesis and is essentially absent in all normal adult human tissue but is strongly expressed in the vasculature of most aggressive tumor types. Antibodies to this target have been shown to selectively localize to the tumor neo-vasculature in animal models and patients with cancer (Villa, Trachsel et al. 2008). Although these antibodies are not therapeutic themselves they are being used as carriers for other potent anti-tumor agents such as I^{131}, IL2 , interferon-α and TNF**.**

The ability to deliver cytokines, growth factors and other immunomodulators specifically to the tumor is a great advance in targeted therapeutics. Interferon-α is currently used in the clinic to treat some cancer types and exerts its anticancer effects either by direct action on the tumor or by indirect mechanisms effecting the patients immune system. The direct anti-tumor effects involve the inhibition of tumor cell proliferation, down regulation of oncogenes, up regulation of tumor suppressors and avtivating proapoptotic pathways. The immunostimulatory effects of interferon-α play a part in the indirect anticancer properties of interferon-α (Frey, Zivanovic et al. 2011). Interferon-α was the fisrt cytokine to to become approved for clinical use in 1986 in hairy cell leukemia and is widely used today for treatment of malignancies such as hepatitis c and metastatic melanoma and in combination with some anticancer drugs. One of the major limitations of interferon-α use is its short half-life and potent toxicity limiting the ability to increase dose. Using an antibody targeting the tumor vasculature and tagging it with interferon-α allows for specific tumor targeting with the cytokine, avoiding healthy tissue and without the high doses and subsequent toxicity of conventional interferon-α treatment. This potential new treatment could help further enhance current cancer treatments strategies (Frey, Zivanovic et al. 2011). The conjugation of bio-active agents such as IL-2 is a promising area in cancer treatment. There has been great success in conjugating the cytokine IL-2 to antibodies with an EDB-IL2 (L19-IL2) antibody currently in stage II clinical trials. L19-IL2 also shows high efficacy in lymphoma models active both as a monotherapy and very potent when used in combination with the anti-CD20 mAb rituximab (Schliemann, Palumbo et al. 2009).

4. Summary

The history of cancer research has shown us that highly inventive and innovative approaches like the use of model organisms such as bacteria to study fundamental mechanisms of DNA repair, the use of worms to study programmed cell death, and the use of yeast to study the cell cycle has identified fundamental hallmarks of cancers (Hanahan and Weinberg 2011). However, the by-pass of the immune system in human cancer development and the realization that many cancer genes are only expressed in vertebrates has placed more pressure on developing more physiological models to identify anti-cancer drug targets. We have reviewed here some of the key targets identified from exploiting our knowledge of the human physiology of cancer, the use of monoclonal therapeutics as a growing innovation in cancer treatment, and the use of mass spectrometry in conjunction with clinical approaches to develop more physiological therapeutic strategies.

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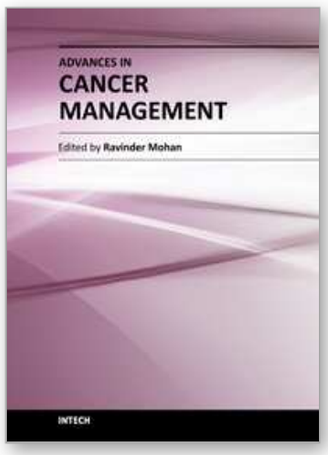

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Cancer is now the most common cause of death in the world. However, because of early diagnosis, better treatment, and advanced life expectancy, many cancer patients frequently live a long, happy, and healthy life after the diagnosis- and often live as long as patients who eventually do not die because of cancer. This book presents newer advances in diagnosis and treatment of specific cancers, an evidence-based and realistic approach to the selection of cancer treatment, and cutting-edge laboratory developments such as the use of the MALDI technique and computational methods that can be used to detect newer protein biomarkers of cancers in diagnosis and to evaluate the success of treatment.

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