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Novel proteins highly expressed in tumors identified by a high throughput immunoproteomic approach

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Cancer biomarker discovery is an extremely active research field in both academia and pharmaceutical companies. Several high throughput technologies were applied through the years to identify proteins specifically related to cancerous phenotype. However, most putative biomarkers identified by conventional proteomic/transcriptomic strategies still need to be clinically validated. Here we present a cancer biomarker discovery approach, based on the use of a large library of antibodies raised against recombinant human proteins, to detect, by immunohistochemistry analysis of excised tumor samples, tumor-associated proteins. Starting from the whole human genome, genes encoding proteins predicted as membrane or secreted were selected and then cloned and expressed by high throughput techniques. Recombinant proteins were used to build a polyclonal antibody library (YOMICS®) currently comprising more than 1,700 murine immune sera. The ability of sera to recognize specific targets predominantly present in tumors was assessed by Tissue Micro Array (TMA, a miniaturized immunohistochemistry analysis) performed on tumor tissue samples and healthy controls from pedigreed patients affected by the most common human tumors (including colon, ovary, breast, lung, and prostate cancers). Even if the screening is still in progress, eight antibodies were identified showing high reactivity on a high percentage (ranging from 40 to 95%) of tumor tissues concerning to one or more cancer types, showing negative staining on correspondent normal tissues. The corresponding protein targets, being novel tumor-associated proteins, were validated and then characterized both at cellular and molecular level by analyzing their expression, cellular localization and biological role in a panel of tumor cell lines. Interestingly, three of the above-mentioned proteins are localized at the plasma membrane, while the other ones are intracellular. Furthermore data so far available show that three proteins are involved in cellular processes relevant to tumor development. A reduced expression of EXN4 and EXN7 impairs the invasive phenotype of considered tumor cell lines, while over-expression of EXN1 increases the cell clonogenic phenotype. Finally the newly identified proteins are promising candidates as new tumor biomarkers and could be exploited to develop target-specific drug therapies.

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Soluble Axl (sAxl) from serum defines a subpopulation of patients with breast, colon, lung, and pancreatic cancers

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Background: Axl is a receptor tyrosine kinase involved in cell growth, differentiation, and migration. It is overexpressed in multiple tumor types and has been implicated in cancer progression and metastasis. The extracellular portion of Axl is characterized by immunoglobulin-like and fibronectin type III repeats, in addition to a binding site for its natural ligand, Gas6. Binding of Gas6 results in the autophosphorylation of Axl and in the activation of multiple cytoplasmic substrates, including members of the PI3K/AKT pathway. Additionally, the extracellular portion of Axl undergoes processing by ADAM10 to yield a soluble form of the receptor. It is postulated that this proteolytic cleavage event is a negative feedback mechanism to downregulate activated AXL and to capture free circulating Gas6.
Materials and Methods: An ELISA system was used to examine sAxl levels in conditioned media from cell lines and in serum samples from patients with colon, lung, breast or pancreatic cancer (drawn prior to chemotherapy). Western blotting and realtime PCR were used to determine Axl expression in cell lines.
Results: The average levels of sAxl in the serum were found to be 27.2 ng/mL (n = 23) in healthy control specimens, and for cancer patients, 35.8 ng/mL (n = 10; p = 0.03) in breast, 35.9 ng/mL(n = 10; p = 0.059) in colon, 40.5 ng/mL (n = 10; p = 0.086) in lung, and 43.1 ng/mL (n = 16; p = 0.012) in pancreas. sAxl levels in tumors averaged at least 31% higher than the sAxl levels in healthy control specimens. Interestingly, in each tumor-specific group there were several (approx. 1 in 4) specimens with extremely high levels of sAxl. Furthermore, sAxl in the conditioned media from pancreatic cell lines was differentially expressed across the panel, and showed good correlation to expression of the Axl receptor at the RNA and protein levels in these cell lines.
Conclusions: We demonstrate that sAxl can be readily detected in cell culture media and in human serum. sAxl levels strongly correlate with Axl kinase expression and sAxl levels are elevated in some cancer patients. Efforts are currently underway to better understand correlations between sAxl expression and other parameters, such as treatment response, overall survival, and histological differences in these patients.

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Looking for a stemness signature in prostate cancer

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Background: Recent data suggest that tumor initiation and growth might be driven by a rare population of cells endowed with stem-properties, and therefore defined as Cancer Stem Cells or Cancer-initiating Cells (CIC). This model might explain the inefficiency of specific anti-cancerous therapies, most of them targeting the bulk of the tumor and not specifically these cancer-initiating cells. CIC may have properties similar to those of normal stem cells, such as self-renewal and differentiation potential and could also share some molecular pathways with embryonic pluripotent stem cells. In this study, we aimed at evaluating the expression of pluripotency and stemness-associated genes in PCA cell lines and prostatic tissues, including PCA and Benign Prostatic Hyperplasia (BPH).
Material and Methods: The study was performed on PC3, Du145 and LNCaP PCA cell lines and on prostate clinical specimens, including PCA and BPH. Expression of specific genes, such as CD133, Oct4, Nanog, Sox2, Klf4, c-Myc, and Chromogranin A (CgA) was evaluated by quantitative real-time PCR. Protein expression was assessed by FACS analysis, or immunofluorescence. Additionally, tumor samples were collected and used for a tissue microarray (TMA), assessing the expression of CgA and Klf4.
Results: PCA cell lines were found to express the specific pluripotency related factors, Oct4, Nanog, Sox2, Klf4, and c-Myc, highlighting the possible presence of pluripotent cells within tumorigenic prostate cell lines. Moreover, the same genes were also well expressed in almost all clinical samples investigated. Notably, there was a trend toward an increased expression of all pluripotency-related genes in tissues from patients bearing PCA, as compared to BPH tissues. In particular, c-Myc and Klf4 genes were significantly more expressed in cancer tissues as compared to BPH and showed a close relationship in their expression as well as with the other pluripotency-related factors. Moreover, analysis of the TMA revealed that Klf4 protein was specifically localized in rare scattered cells within the basal compartment, morphologically similar to neuroendocrine cells. Supporting these results, a high correlation was found between Klf4 and CgA expression.
Conclusions: Taken together, these results indicated the presence of a specific stemness signature in prostatic tissues. In particular, Klf4 gene was significantly more expressed in cancerous tissues as compared to BPH samples. Additionally, Klf4 protein appeared to be expressed by a sub-population of neuroendocrine cells within the prostate, highlighting the potential importance of these cells in prostate cancer progression.

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Level of c-kit expression on pre therapeutic mediastinal lymph node biopsy does not predict its level of expression on post chemotherapy lung tumor

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Background: Experimental evidence suggests that stem cell factor (SCF) and its receptor c-kit (CD117) play an important role in survival and proliferation of lung cancer stem cells (CSC), providing a promising target to neoadjuvant treatment of non-small cell lung cancer (NSCLC). However, efficacy of c-kit inhibitors relies on c-kit expression on the primary tumor, and some patients with N2 NSCLC are diagnosed on the basis of mediastinal lymph node biopsy. Therefore, we sought to determine whether the level of c-kit expression on pre therapeutic mediastinal lymph node biopsy predicts its level on lung primary tumor.

Material and Methods: We set a retrospective study including 13 patients with resectable NSCLC and N2 involvement. All patients underwent pre therapeutic mediastinal lymph node biopsy, neoadjuvant cisplatin-based chemotherapy, and complete resection of the primary tumor associated with mediastinal lymph node dissection. C-kit expression was measured by immunohistochemistry on lymph node biopsies and primary lung tumors. Immunopositive cells were counted and expressed as a percentage of tumor cells. The intensity of immunostaining was categorized as follows: 0, negative; +, low; ++, moderate; and +++, high.
Results: On pre therapeutic mediastinal lymph node biopsy, c-kit expression was found in one patient (1/13 = 7%), quantified as 30% of tumor cells, with low immunostaining intensity. On post chemotherapy lung tumor, c-kit expression was detected in 4 patients (4/13 = 30%), in 5 to 100% of cells, with low to high immunostaining intensity. Difference in c-kit expression between pre therapeutic mediastinal lymph node biopsy and post chemotherapy lung tumor was significant (McNemar chi-square test, P = .0455). Interestingly, the four patients with positive post chemotherapy lung tumor had negative pre therapeutic mediastinal lymph node biopsy.
Conclusion: Level of c-kit expression on pre therapeutic mediastinal lymph node biopsy does not predict its level of expression on post chemotherapy primary lung tumor. Additional studies should determine whether this discrepancy is linked to tissues heterogeneity or to neoadjuvant treatment.

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Organic anion transporting polypeptides contribute to prostate cancer progression

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The OATP family of transporters has been previously studied for their roles in drug elimination and pharmacodynamics. Recently, polymorphisms in family members have been implicated in prostate cancer disease progression and survival. The current hypothesis is that they effect clinical outcome by transporting growth hormones or chemotherapy agents. However, little is known about the prevalence and cause of OATP expression in cancer. Therefore, we completed a study on 321 primary tissue samples from 21 normal and cancerous patient samples examining the expression of three family members implicated in cancer; OATP1B3, OATP1B1 and OATP2B1. The results showed that OATP2B1 is more ubiquitously expressed in all tissues than OATP1B1 and OATP1B3. Based on expression frequency, OATP2B1 could be significant in or used as a biomarker for lymphatic and thyroid cancer. In contrast, OATP1B1 is expressed exclusively in some primary cancer tissue samples, but at a much lower frequency (<20% in 8 cancers) than both OATP1B3 and OATP2B1. OATP1B3 was also expressed in fewer normal tissue types, however it was expressed in 50% of cancerous prostate samples and there is a trend of increasing OATP1B3 expression with higher Gleason score. This supports previous data suggesting a role of OATP1B3 in advancing prostate cancer. Further experiments from quantitative PCR and western blot suggest that hypoxia elements in the OATP1B3 promoter are activated under tumor conditions and explain the increased expression in tumor cells. In addition, transport studies in *Xenopus* oocytes showed that optimal transport occurs at low androgen levels such as those seen in patients after androgen deprivation therapy. In summary, upon examining the expression of OATP1B3, OATP1B1, and OATP2B1 in primary tissue samples, there appear to be several cancers for which they may be progression or cancer biomarkers and warrant further study.

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Phosphorylated histone H3 and S6 proteins as biomarkers for targeted anti-cancer drug action measured using a combined IHC/Western method in skin biopsies

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In recent years there has been a rapid increase in targeted tumor therapy approaches. In contrast to classic chemotherapy, which non-specifically targets proliferating cells, these novel approaches attempt to specifically interfere with processes thought to be crucial for the tumor cell survival. Nevertheless, despite extensive validation, an immediate effect of target interference on tumor growth may not necessarily be expected. Thus, in order to demonstrate activity of these substances, a marker known to be modified upon inhibition of the target may serve as a surrogate to tumor inhibition. Taking in consideration the difficulties to perform sequential tumor biopsies, the use of surrogate tissues like blood or skin is being explored instead. Blood is relatively easily accessible, but unlike skin tissue, blood may not reflect the fact that drugs have to penetrate multiple layers of tissue in order to reach their target in the tumor. Also, other than cells in the dermis, which still proliferate, peripheral blood cells have largely exited the cell cycle. Effects of anti-mitotic drugs may therefore not easily be demonstrated. We have therefore developed a method which reproducibly allows the detection and quantification of potential target proteins via Western Blots and immuno-histochemistry (IHC) from human skin biopsy halves.
We looked at two phosphorylated marker proteins, phospho-histone H3, and phospho-S6. Histone H3 is phosphorylated on Ser 12, mainly by aurora B, which is a bona fide cancer drug target. It is thus a good marker for inhibition of aurora B kinase activity, but may also serve, albeit in a less direct manner, as a marker for cells arrested in M-phase, since this phosphorylation event is closely linked to chromosome condensation. S6 is the downstream target of p70S6K, which in itself may be a drug target, but lies downstream of AKT and mTOR kinases, both of which have been, and still are, exploited as drug targets.
In order to evaluate the above biomarkers, we employed this method using skin biopsies of mice treated with a variety of kinase inhibitors, i.e. Nexavar, Tozasertib, Sunitinib, and Everolimus. Data will be presented on the effect of H3 and S6 phosphorylation. Furthermore, data on the measurement of these proteins, and the stability of the signals in human biopsies from reduction surgery skin folds will be presented.
This is a versatile method to measure biomarkers to characterize the specificity of novel targeted drugs.

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Signaling pathways contributing to head and neck carcinoma radioresistance

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Radiation therapy plays an important role in the management of head and neck carcinoma. However, the problem of radioresistance and molecular mechanisms by which head and neck cancer cells overcome cytotoxic effects of radiation therapy remains to be elucidated. In order to investigate possible intracellular mechanisms underlying the head and neck cancer recurrences after radiotherapy, we have established three radiation-resistant squamous cell carcinoma cell lines, CAL27-IRR, SCC25-IRR and FaDu-IRR derived from the parental CAL27, SCC25 and FaDu head and neck cancer cells by repetitive exposure to ionizing radiation (summary dose was 100 Gy). CAL27-IRR, SCC25-IRR and FaDu-IRR (IRR cells) demonstrated pronounced radioresistance, enhanced oxygen consumption and activated epidermal growth factor (EGF) receptor related pathways, such as Ras-MAPK and PI3K-Akt and Jak-STAT. In order to elucidate additional mechanisms involved in the radioresistance development and increased oxygen consumption, we determined differences in the proteome profile of parental and IRR cells using two-dimensional differential gel electrophoresis (2-D DIGE) followed by computational image analysis and mass spectrometry. It was found that identified proteins were involved in the regulation of intracellular routs providing cell survival, release of angiogenesis-related factors, increased motility and invasiveness, enhanced mutagenesis, DNA repair and regulation of glycolysis. Our data suggest that some of the found proteins could be considered as potential biomarkers of head and neck cancer radioresistance and/or targets to improve radiotherapy outcome.

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External quality control for companion molecular diagnostics using cell lines

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Background: The use of companion diagnostics to ensure that cancer patients get optimal treatment is increasing, yet the development and implementation of such diagnostics often lags behind the development of new anti-cancer drugs. A prime example is the use of EGFR and KRAS mutation testing in lung and colorectal cancer respectively. Implementation to good laboratory practice standards is usually a requirement for laboratory accreditation and this needs quality assurance – both internal and external. The use of human tumour samples is difficult for many reasons, not least because of the variation inherent in such samples which renders them relatively poor controls. We have therefore developed a method using cell lines for a national external quality assurance scheme (NEQAS) which is also suitable to assist development of the tests themselves.
Materials and Methods: Cell lines containing specific EGFR and KRAS mutations were obtained from Horizon Discovery Ltd (Cambridge, UK) and passaged 2−3 times until sufficient cells were available to make fibrin (cytoclot) or agar embedded cell pellets. These were embedded in paraffin