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Impact of Upfront Cellular Enrichment by Laser Capture Microdissection on Protein and Phosphoprotein Drug Target Signaling Activation Measurements in Human Lung Cancer: Implications for Personalized Medicine

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Abstract

Purpose—The aim of this study was to evaluate whether upfront cellular enrichment via laser capture microdissection is necessary for accurately quantifying predictive biomarkers in non-small cell lung cancer tumors.

Experimental design—Fifteen snap frozen surgical biopsies were analyzed. Whole tissue lysate and matched highly enriched tumor epithelium via laser capture microdissection (LCM) were obtained for each patient. The expression and activation/phosphorylation levels of 26 proteins were measured by reverse phase protein microarray. Differences in signaling architecture of dissected and undissected matched pairs were visualized using unsupervised clustering analysis, bar graphs, and scatter plots.

Results—Overall patient matched LCM and undissected material displayed very distinct and differing signaling architectures with 93% of the matched pairs clustering separately. These differences were seen regardless of the amount of starting tumor epithelial content present in the specimen.

Conclusions and clinical relevance—These results indicate that LCM driven upfront cellular enrichment is necessary to accurately determine the expression/activation levels of predictive protein signaling markers although results should be evaluated in larger clinical settings. Upfront cellular enrichment of the target cell appears to be an important part of the

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Conflict of Interest

The authors are inventors on US Government and University assigned patents and patent applications that cover aspects of the technologies discussed such as Laser Capture Microdissection and Reverse Phase Protein Microarrays. As inventors, they are entitled to receive royalties as provided by US Law and George Mason University policy. MP, LL, and EP are consultants to and shareholders of Theranostics Health, Inc.

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workflow needed for the accurate quantification of predictive protein signaling biomarkers. Larger independent studies are warranted.

Keywords

Individualized therapy; Laser Capture Microdissection; Non Small Cell Lung Cancer; Reverse Phase Protein Microarray; Pathway activation mapping

1. INTRODUCTION

Lung cancer is the leading cause of cancer related mortality worldwide among both men and women and non-small cell lung cancer (NSCLC) represents the most common type of pulmonary malignancy accounting for approximately 85% of all cases [1].

Historically, NSCLC has been treated with a combination of surgery, platinum-based chemotherapy and/or radiation therapy where the combination of the different therapeutic options was dictated by the stage of the tumor at the initial diagnosis or its progression.

The discovery of genetic and epigenetic alterations involved in the onset and progression of malignant lesions and the development of treatment targeting the product of these deranged genes appear to be promising for managing NSCLCs more successfully. However, while selection of many of these targeted agents is currently performed using genomic/genetic based predictive markers, the drugs themselves (many of them kinase or enzyme inhibitors) work specifically by modulating the activated protein drug target. The introduction of anti-epidermal growth factor receptor molecules for example is historically considered a major turning point for the treatment of NSCLC harboring EGFR mutations [2,3]. Similarly in tumors presenting with the echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) translocation, or amplification on the hepatocyte growth factor receptor (MET), the use of targeted agents against the deranged proteins have led to significant improvement in controlling the progression of the disease and in developing targeted compounds [4-6]. As a consequence a number of tyrosine kinase inhibitors and monoclonal antibodies targeting EGFR, ALK and VEGF were approved for the treatment of lung cancer and numerous compounds targeting HER2, IGF-1R, c-Met, HSP90 and PDGFR are currently under investigation (Table 1).

Patients' stratification to targeted treatment based on their molecular profile has shown to be a condition sine qua non to achieve higher response rates when assigning patients to tailored treatment [7-10]. In this context, the development of high-throughput multiplex technologies able to accurately measure these molecular derangements are constantly tested as potential companion diagnostics for stratifying patients to the most appropriate treatment.

Reverse phase protein microarray (RPPA), a multiplex proteomic platform, has shown unique and very promising abilities for identifying prognostic and predictive markers, measuring the phosphorylation/activation state of hundreds of proteins at once from microscopic quantities of tissue, as well as stratifying patients to personalized treatments [11-14]. Indeed, this high-throughput multiplex platform allows for broad investigation into the changes in the signaling architecture of the malignant cells starting from very small

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amounts of biological material such as a core needle biopsy. Moreover, by constructing each array with built in standard curves and controls, the RPPA platform is currently capable of delivering quantitative information that can be easily used for stratifying patients for individualized treatment [15,16].

In a number of published studies, RPPA along with a number of genomic techniques has been coupled with upfront sample enrichment using Laser Capture Microdissection (LCM) to overcome the problem of tissue heterogeneity and to improve accuracy by measuring proteomic and genomic changes directly within the malignant cells [17]. Recent publications have shown that LCM-based protein/phosphoprotein measurements correlate more closely with independently obtained measurements of the same analyte using FDA approved technologies such as IHC and FISH (for HER2 determination) compared to undissected material [18], and produce more accurate correlation with genomic derangements such as PTEN loss and EGFR phosphorylation in TCGA (The Cancer Genome Atlas) glioblastoma samples [19]. Through the isolation of pure cell populations from the surrounding microenvironment, LCM has revolutionized the analysis of complex tissues since the representation of the different cell populations differs greatly across individuals [20].

This pilot study evaluated the role of LCM coupled with RPPA in the quantification of the expression/activation levels of the proteins that are the major drug targets for targeted therapies currently under investigation for NSCLCs. NCLSC tumor epithelial cells were isolated by LCM and the protein drug target activation profile of the microdissected samples was compared to the matched undissected tissue lysate obtained from the same contiguous sample in order to investigate whether the drug target signaling architecture from purified tumor cells is substantially different from the whole tissue sample.

2. MATERIALS AND METHODS

2.1 Sample collection

A total of 15 archived adenocarcinomas of the lung were used for this study. Samples were originally collected during surgery at the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL) and at the S. Maria della Misericordia Hospital (Perugia, Italy) following standard ethical practice. Informed consent was collected from each participant before undergoing surgical procedure. Samples were frozen in liquid nitrogen within 30 minutes from collection in order to minimize pre-analytical variables of protein/phosphoprotein loss, and within the time period previously found to retain in vivo signaling states [21] and subsequently embedded in Optimal Cutting Temperature compound (OCT).

2.2 Sample preparation

Eight μm serial sections were cut and mounted on uncharged microscope glass slides. For each sample a total of 6 slides were prepared for this analysis. The slide in the middle of the set was stained with hematoxylin and eosin to estimate the amount of tumor present in the specimen. Unstained slides were stored at –80°C until processed using Laser Capture Microdissection (LCM) or directly lysed to obtain whole tissue lysate (TL).

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Briefly, each section was fixed in 70% ethanol, rinsed in dH2O, stained with hematoxylin and Scott's tap water, and dehydrated using an increasing series of alcohol (70%, 95%, and 100% EtOH respectively) and xylene. All solutions, except for the 95-100% EtOH and xylene, were supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN). After staining the sections were dried at room temperature for few seconds, and pure cancer cells were immediately captured using a Veritas Microdissection system (Arcturus Bioscience, Mountain View, CA, USA). For each specimen approximately 7,000–15,000 cells were collected on CapSure Macro LCM caps (Arcturus Bioscience, Mountain View, CA) (Supplementary figure 1). Caps were lysed using a 1:1 solution of 2× Tris-Glycine SDS Sample buffer (Invitrogen Life Technologies, Carlsbad, CA) and Tissue Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with 2.5% 2-β-mercaptoethanol (Sigma, St. Louis, MO).

One of the slides sequential to the one stained with hematoxylin and eosin and contiguous with those used for LCM was designated for the direct protein extraction. Undissected tissues were fixed, stained, and lysed using the same procedure followed for the LCM samples. All samples were boiled for 8 min and stored at −80°C until further analyzed.

2.3 Reverse Phase Protein Microarray (RPPA)

LCM samples and matched whole tissues lysates (TL) were printed in triplicate onto nitrocellulose coated slides (Grace Bio-labs, Bend, OR) using an Aushon 2470 arrayer (Aushon BioSystems, Billerica, MA). Before proceeding with immunostaining, each array was treated with Reblot antibody stripping solution (Chemicon, Temecula, CA) for 15 minutes and blocked in I-block solution (Tropix, Bedford, MA) for one hour to reduce non-specific binding. Each array was probed with one primary antibody on an automatic Autostainer (Dako Cytomation, Carpinteria, CA) using the Catalyzed Signal Amplification System kit (CSA; Dako Cytomation, Carpinteria, CA). Samples were probed for a total of 26 analytes that are either direct targets or downstream effectors of drugs currently approved or under investigation for the treatment of NSCLC (Supplementary table 1). Antibody specificity was tested as previously described [22]. Fluorescent detection was achieved using the streptavidin-conjugated IRDye680 (LI-COR Biosciences, Lincoln, NE) according to the manufacturer's instructions. The total amount of protein contained in each sample was measured by Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR) as previously described [23]. Images were acquired using the PowerScanner (TECAN, Mönnedorf, Switzerland) and spot intensity values were quantified using MicroVigene software Version 5.1.0.0 (Vigenetech, Carlisle, MA) as previously described [23].

2.4 Statistical analysis

To evaluate the overall signaling network of dissected and undissected samples unsupervised hierarchical clustering analysis using the Ward's method was performed in JMP 5.1 (SAS Institute Inc., SAS, Cary, NC).

To compare the intensity values of the dissected and undissected samples, scatter plots were generated in GraphPad Prism software Version 6 (GraphPad Software Inc, San Diego, CA). Because the TLs had lower and compressed values compared to the LCM samples, for each

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analyte of interest dissected and undissected samples were first normalized to the respective population median (LCM or undissected). Samples were then rank-ordered based on the activation levels of the normalized LCM samples and color-coded based on their quartile distribution. Pairwise comparison was performed to investigate changes in terms of quartile switching between dissected and undissected material collected from the same sample. By rescaling both LCM and TLs on the median value of the population, we were able to directly investigate whether upfront cellular enrichment via LCM is necessary for accurately measuring the activation level of drug targets within cancer cells compared to the information obtained from the TLs. This approach allowed to evaluate whether patients that presented with the highest activation level of the target of interest in the LCM material showed the same characteristics also in the TLs. Similarly, for a selected number of endpoints, bar graphs were used to show the percentage of patients for which the dissected and undissected material showed a difference in the distribution equal or greater than 2 quartiles. Finally, bar graphs were created to evaluate the fold changes in the activation levels of a number of analytes in dissected and undissected material in relationship to the amount of primary tumor. Each TL sample was normalized to the matched LCM. Mean and SEM were shown for comparison.

3. RESULTS

A total of fifteen matched dissected and undissected adenocarcinomas of the lung were used for this study. Tumor percentage estimates were performed independently by two operators (EB, MP) trained and overseen by a board-certified pathologist (LL). Based on tumor percentage, sample were classified as <40% (n= 2), 50% (n= 5), 60% (n= 2), 70% (n= 4), and >70% (n= 2).

Unsupervised hierarchical clustering analysis was used to broadly compare the signaling architecture of laser captured enriched samples and matched whole tissues. As shown in figure 1A, the signaling profile of microdissected material strongly differed from the one obtained from matched undissected samples. Overall LCM and whole tumor lysate (TL) material had the tendency to create 2 separate clusters with the vast majority of the TL showing an increased activation of a number of receptor tyrosine kinases (RTKs) including, but not limited to PDGFR Y751, VEGFR Y951 and Y996, along with an overexpression and activation of mTOR. On the contrary, a subgroup of LCM samples showed an increase activation of HSP90 T5/7, EGFR Y992, and Mek S298. Overall, only one matched LCM-TL pair (the sample with the highest percentage of tumor epithelium, 70%) clustered together (1/15, 7%).Even then the two samples were not included in the same branch of the dendrogram and showed some differences in the activation of a number of RTKs (Figure 1B).

To further investigate the variability in terms of expression/activation of the drug targets between LCM preprocessed samples and matched TL tissues, a pairwise comparison was performed. For each analyte evaluated in the study the range of the intensity values for LCM samples and TLs was reported in Supplementary table 2. Scatter plots of the LCM and the matched TL values were created for EGFR, IGFR, MAPK, AKT-mTOR pathway components as well as ALK (all proteins that are of central importance to a number of

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targeted agents for NSCLC) (Figure 2 and Supplementary figure 2 for raw data). Color coding of the LCM samples by quartiles was performed to re-orient the continuous variable RPPA data into more familiar immunohistochemistry-like determinants. Systemic changes in the rank order were found for the activation levels of a number of key proteins highly deregulated in lung cancer such as EGFR Y1148 and IGF-1R Y1135/IR Y1146 where 40% of the samples showed a difference equal to or greater than two quartiles between the LCM and the TL samples (Figure 2- Figure 3A),. Similarly, quartile distribution differences were found for 40% of the LCM-TL samples for VEGFR Y951 and 27-30% for a number of the EGFR family members and Ret Y905 (Figure 2 and 3A). On the contrary the activation of Erk1/2 T202/Y204 in the LCM samples was mostly conserved in the matched TL samples indicating that the need for upfront enrichment varies between different analytes and it requires deeper investigation (Figure 2 and Figure 3A). Analysis of LCM-TL differences for the overall protein expression of some of these drug targets was evaluated (Figure 3B), and revealed similar discordance between the data obtained from TL and LCM material.

Finally, for a selected number of samples, the impact of the starting amount (% tumor epithelium) of tumor cells on the signaling network activation measurements was evaluated by comparing the activation level of a number of protein drug targets in the TL compared to the LCM samples. Our data reveal that a higher starting amount of tumor epithelium often did not result in more accurate signaling data derived from the cancer cell alone indicating that the subpopulation of cells in the surrounding microenvironment might differ from sample to sample and might strongly impact the overall cellular signaling measured (Figure 4).

Finally, using an independent set of five NSCLC tissue samples we evaluated whether the LCM methodology itself affects the expression/activation level of the analytes of interest. For each specimen three different lysates were prepared: 1) pure tumor cell populations collected via LCM, 2) TLs prepared as described above and 3) microdissection of the entire tissue section (LCM-TL). RPPA was performed to analyze the expression/activation levels of 15 analytes measured in the study.

Unsupervised hierarchical clustering analysis was used to evaluate whether the different phases on the LCM procedure have an impact on the final findings. As shown in figure 5, for all five pairs analyzed the TLs and the matched LCM-TL were contained within the same cluster, indicating that the LCM process itself does not impact the RPPA data. On the contrary in all 5 of the tumor samples, the RPPA signaling data derived from the LCM procured epithelium was found on a different branch of the dendrogram indicating that the signaling architecture of the tumor epithelium is different. This difference is based on the cellular input and not on the LCM process itself.

4. DISCUSSION

Utilization of molecularly tailored agents for the treatment of NSCLCs has significantly impacted the response rate of lung cancer patients. Since accurate stratification and patient selection to individualized treatments is becoming of primary importance for efficacious therapeutic regimens, there is an urgent need to accurately measure the expression and

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activation levels of biomarkers with predictive significance. Due to the important role that signaling proteins have in tumorigenesis and metastatic progression, and concomitant with the fact that nearly all of the FDA-approved and experimental therapeutics used for treatment of NSCLC (Table 1) work by modulating protein network activation/phosphorylation, proteomic tools and phosphoprotein based pathway activation analysis are becoming more frequently used alongside genomic platforms to identify potential therapeutic targets and responsive patients. Recently, a number of studies in patients with NCSLC have highlighted the importance of measuring phosphoproteins and activated protein signaling networks for accurate patient selection. Indeed, phosphorylation/activation of EGFR protein in NSCLC patients with wild type EGFR was shown to be highly predictive of response to erlotinib as well as overall response rates of NSCLC patients to EGFR directed therapies [24,25]. In this context new high-throughput multiplexed proteomic technologies such as RPPA that can quantitatively measure the activation state of dozens to hundreds of key signaling proteins from microscopic quantities of tissue, such as from a needle biopsy, have shown encouraging results in providing both precise and quantitative measurement of predictive markers as well as functional classification of NSCLC [12]. Moreover, the RPPA derived data for important markers such as HER2, when compared to data derived from FDA-approved diagnostic tests such as IHC and FISH, showed concordance greater than 90% [18,26]. The intra- and inter-assay reproducibility of the RPPA has been previously determined. Coefficient of variation within the same array was shown to be between 5.0 and 18.1%, and reproducibility between arrays was within 4.0-17.8% [21,27]. Moreover, as recently demonstrated by Mueller and colleagues, concordance between genomic alterations and protein expression/activation is more accurate when samples undergo LCM [19]. This may be due to the ubiquitous expression of most of the proteins in many different cellular subtypes such as adipocytes, immune cells and other stroma components [28,29]. This study assessed the role of RPPA coupled with LCM in improving the accuracy and reliability of measuring key drug target proteins and phosphoproteins for NSCLCs.

In this study, LCM and matched undissected samples were concomitantly analyzed to evaluate the expression/activation level of a number of analytes that are the direct targets or downstream effectors of therapeutic compounds currently in use for the treatment of NSCLCs. Overall this study provides evidence that upfront tumor cell enrichment may be a necessary requirement to accurately measure predictive targets when analyzing clinical samples wherein the data generated is used for patient-by-patient selection and stratification for therapy assignment (so-called companion diagnostic markers). These results are in line with previous studies demonstrating that the isolation of pure cell populations has a significant impact in providing more precise results when using both genomic and proteomic techniques [17-19, 29-34]. Indeed, as shown in the unsupervised analysis, LCM-preprocessed samples and matched whole tissues lysates were contained within distinct clusters, indicating that overall the quantification of the drug targets and their activation varies enormously based on whether or not the tumor cells were enriched by LCM. Similar results were previously reported by Wulfkuhle and colleagues on breast cancer specimens and by Silvestri et al. on colorectal cancer indicating that the need for cellular enrichment transcends the localization and the origin of the tumor [18,29].

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Since EGFR represents a promising target for the treatment of NSCLC and since it is well known that EGFR activating mutations and/or protein activation are robust predictors of sensitivity to TKIs [24], we then evaluated the activation level of EGFR and its downstream substrates (such as MEK and Erk) individually in matched LCM and TL samples. The results revealed high differences in the activation of EGFR and MEK in samples that were processed using LCM. The discrepancy between LCM pre-processed samples and TL was not reduced by increasing the total amount of tumor. Similar results were reported by Mueller and colleagues indicating that information about the signaling network is lost when the tissue is processed as a whole [19].

Due to the relatively low number of samples included in this analysis, this data should be considered a pilot and inaugural investigation and these results will need to be evaluated in the context of larger study sets. Nevertheless the clear and distinct differences in protein signaling activation measurements seen between LCM and undissected patient-matched samples even in these small numbers of patients provides clear justification for the use of a LCM-based workflow as a fundamental aspect of molecular analysis wherein the protein/phosphoprotein data produced is used for patient selection and therapy assignment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EtOH	Ethanol
FDA	Food and Drug Administration
LCM	Laser Capture Microdissection
MAb	Monoclonal Antibody
NSCLC	Non Small Cell Lung Cancer
OCT	Optimal Cutting Temperature compound
RPPA	Reverse Phase Protein Microarray
RTK	Receptor Tyrosine Kinase
TL	whole Tissue Lysate

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Statement of clinical relevance

In the era where molecular information is becoming a companion diagnostic tool for allocating cancer patients to personalized treatment, the identification of techniques that provide accurate determination of the stratifying biomarkers levels is of central importance. Since the tumor microenvironment is very heterogeneous in terms of cell composition and the subpopulation of cells varies greatly across patients, when tumor specimens are analyzed without considering this important variable, inaccurate conclusions about the level of any given predictive/prognostic marker might be drawn. Here we evaluated whether upfront cellular enrichment of tumor epithelium via laser capture microdissection impacted the determined measurements of the activation/phosphorylation levels of important key signaling proteins centrally implicated as major drug targets/companion diagnostic makers in non-small cell lung cancer (NSCLC) patients. To this end the activation/phosphorylation levels of 26 drug targets and downstream substrates were measured in matched dissected and undissected NSCLC samples. Overall our data revealed a substantial difference at the signaling level of undissected and matched dissected pairs indicating that in order to accurately quantify predictive markers for cancer patients, it is of primary importance to assess and understand the level of the target analyte within the context of the tumor microenvironment and in the subpopulation of cells of interest.

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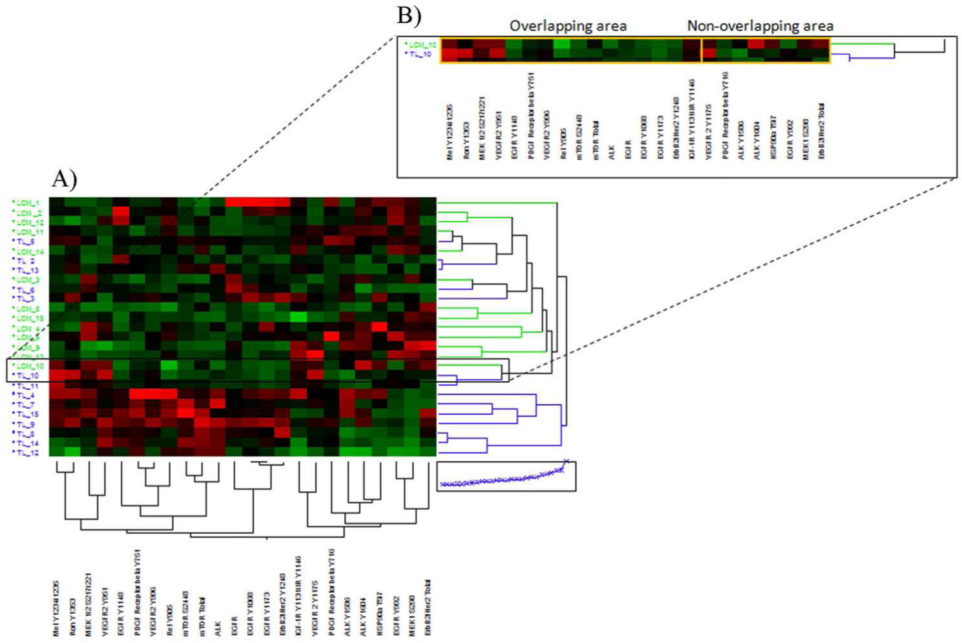

Figure 1.

A) Unsupervised hierarchical clustering analysis of laser capture microdissected lung adenocarcinomas (LCM=green) and patient-matched whole tissues (TL=blue). The activation/phosphorylation levels of 25 signaling proteins that are direct targets of precision therapeutics which are now either used or being evaluated in the treatment of NSCLC were measured and distinct clusters were revealed based on sample preparation method. B) Only for one of the pairs, the LCM material and the matched TL sample were next to each other and showed partial overlapping in the signaling architecture (see yellow rectangles).

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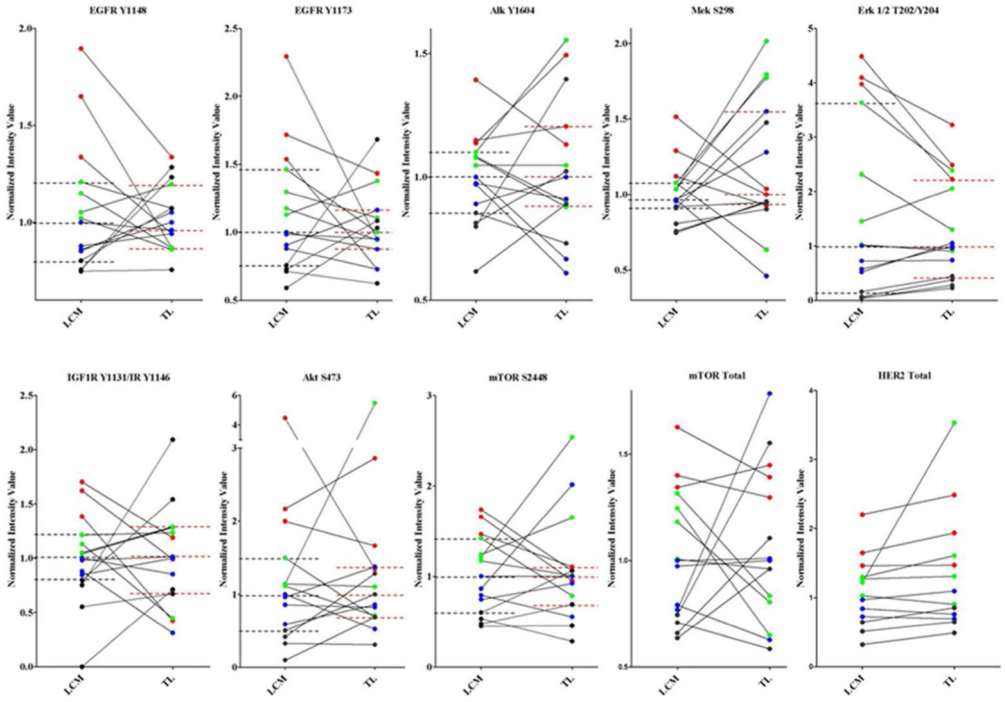

Figure 2.

Pairwise comparison between LCM and matched whole tissues (TL) samples for a number of important drug targets for NSCLC. Samples were first normalized to the median of the population of origin (LCM or TL) and subsequently color-coded based on their quartile distribution (black 1st quartile, blue 2nd quartile, green 3rd quartile and red 4th quartile) in the LCM sample, and then the same patients were ranked in the TL samples. Paired values were connected with solid lines to show the distribution of the matched LCM and TL. Black and blue dashed lines indicate the quartiles for the LCM and TL respectively.

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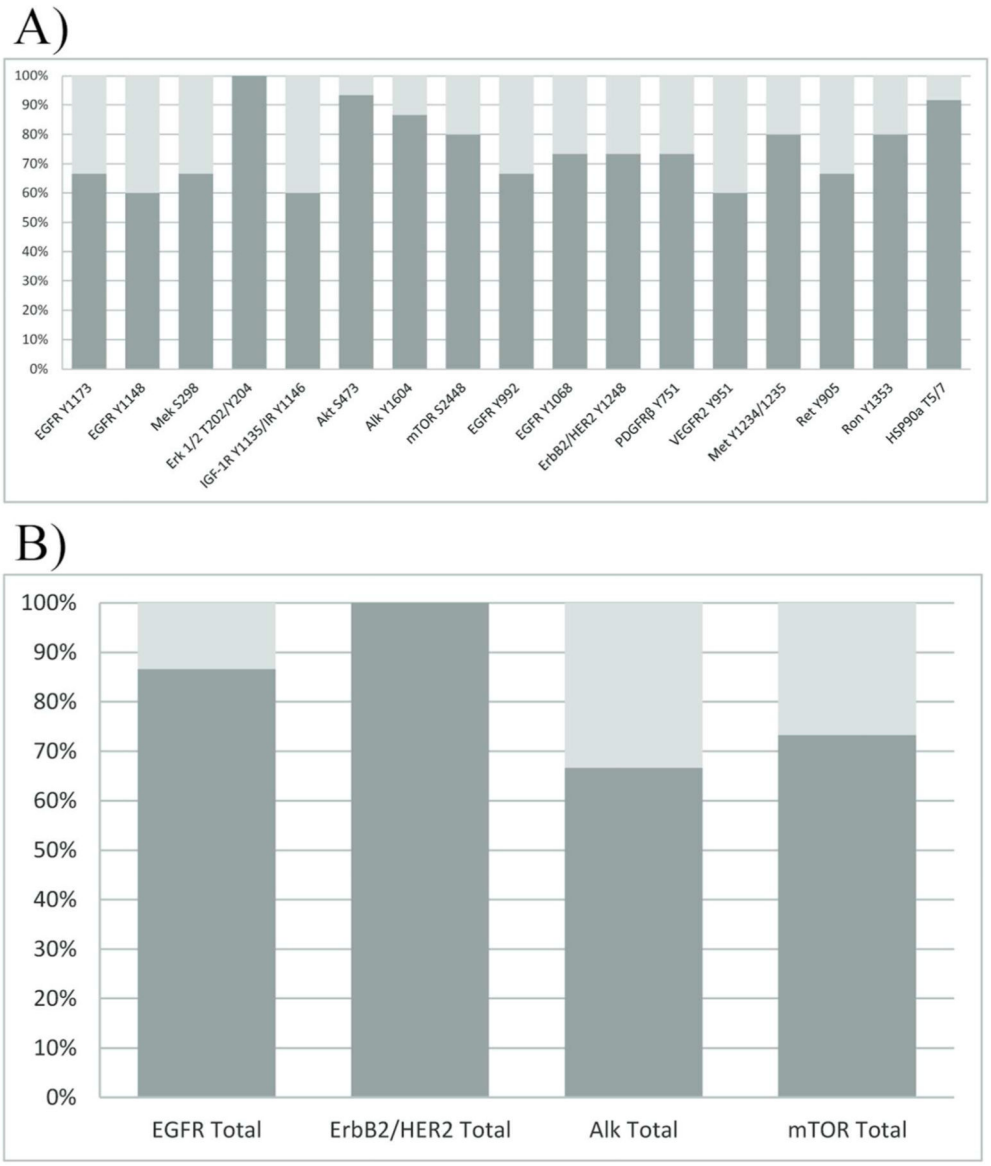

Figure 3.

Evaluation of the proportion of cases in which the TL differs from the matched LCM samples for ≤2 (black bars) or more than 2 quartiles (gray bars). The analysis was conducted for activation/phosphorylation (A) as well as total expression (B) of a number of drug targets.

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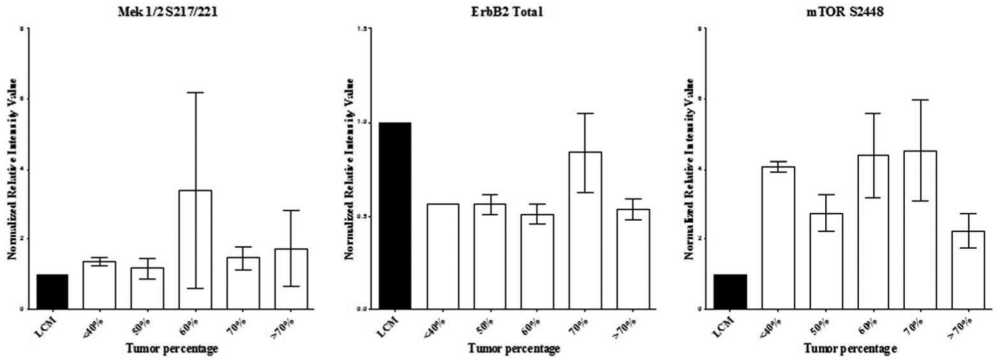

Figure 4.

Fold change between LCM and TL based on the amount of tumor present in the specimen after normalization of each TL to the matched LCM sample. For each group, mean and SEM are shown.

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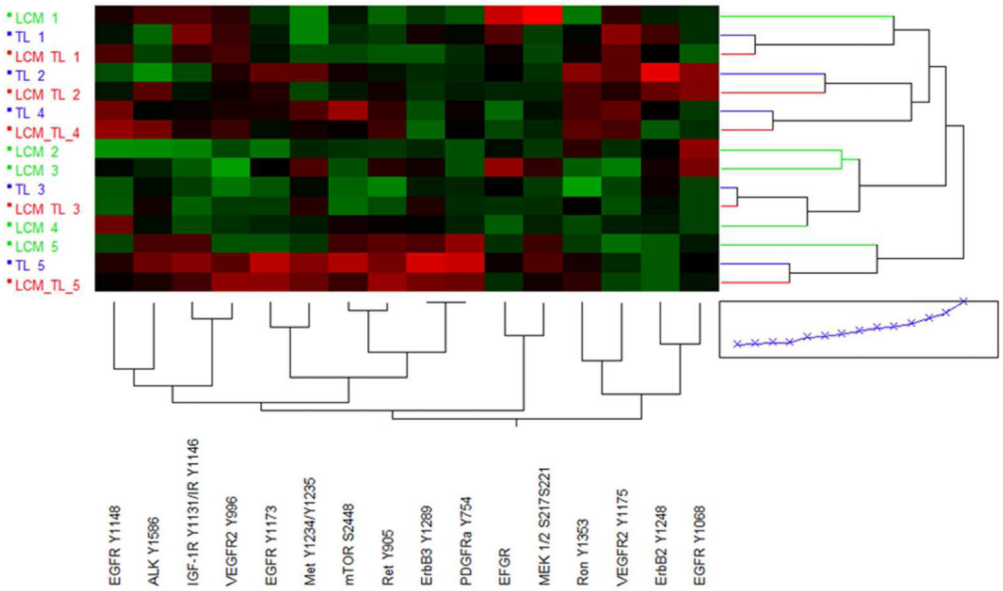

Figure 5.

Unsupervised hierarchical clustering analysis of laser capture microdissected lung adenocarcinomas (LCM=green) matched whole undissected tissues (TL=blue) and LCM of the entire section (LCM-TL=red).

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Table 1

List of FDA approved compounds as well as a selected number currently under investigation for the treatment of NSCLC.

FDA - APPROVED NSCLC TREATMENTS				
Generic name	***Description***	***Drug target***	***Company***	***Indication*** (***http://www.fda.gov/***)
Gefitinib	RTK inhibitor	EGFR	AstraZeneca	First-line treatment for patients with metastatic NSCLC with EGFR exon 19 deletions or exon 21 (L858R) mutations
Afatinib	RTK inhibitor	EGFR	Boehringer Ingelheim	First-line treatment for patients with metastatic NSCLC with EGFR exon 19 deletions or exon 21 (L858R) mutations
Erlotinib	RTK inhibitor	EGFR	Astellas Pharma Inc.	First-line treatment of patients with metastatic NSCLC with EGFR exon 19 deletions or exon 21 (L858R) mutations
Bevacizumab	MAb	VEGF-A	Genentech, Inc	First-line treatment of unresectable, locally advanced, recurrent or metastatic non–squamous NSCLC in combination with carboplatin and paclitaxel
Crizotinib	RTK inhibitor	ALK	Pfizer	Treatment of patients with metastatic NSCLC whose tumors are anaplastic lymphoma kinase (ALK)-positive

NSCLC TREATMENTS UNDER CLINICAL INVESTIGATIONS				
Generic name	***Description***	***Drug target***	***Company***	***Phase*** (***https://clinicaltrials.gov/***)
PF-06463922	RTK inhibitor	ALK/ROS1	Pfizer	Phase I/II
LDK378	RTK inhibitor	ALK	Novartis	Phase I/II/III
Dabrafenib	RTK inhibitor	BRaf	GlaxoSmithKline	Phase II studies
Tivantinib (ARQ 197)	RTK inhibitor	c-Met	ArQule	Phase I/II studies
Onartuzumab	MAb	c-Met	Genentech, Inc	Phase II/III
Cabozantinib (XL 184)	RTK inhibitor	c-Met, VEGFR-2, RET, c-Kit, FLT3 and TEK	Exelixis Inc and Bristol-Myers Squibb Co	Phase I/II
Necitumumab	MAb	EGFR	Lilly	Phase I/II /III
Dacomitinib (PF-00299804)	RTK inhibitor	pan-HER	Pfizer	Phase III
AUY922	RTK inhibitor	Hsp90	Novartis	Phase I/II
Ganetespib	RTK inhibitor	Hsp90	Synta Pharmaceuticals	Phase I/II
Selumetinib (AZD6244)	RTK inhibitor	MEK1/2 and Erk1/2	AstraZeneca	Phase II/III studies
PD0325901	RTK inhibitor	MEK1/2	Pfizer	Phase I/II studies
Trametinib (GSK1120212)	RTK inhibitor	MEK1/2	GlaxoSmithKline	Phase I/II studies
Pazopanib	RTK inhibitor	VEGFR1-3, PDGFRα, PDGFRβ and c-Kit	GlaxoSmithKline	Phase II/III studies
Vandetanib	RTK inhibitor	VEGFR2 and 3, RET and EGFR	AstraZeneca	Phase II/III studies
Sorafenib	RTK inhibitor	VEGFR2–3, PDGFRβ, c-kit, Raf and FLT-3	Bayer	Phase II/III studies
Motesanib	RTK inhibitor	VEGFR1–3, PDGFR, c-Kit and RET	Amgen	Phase II/III
Nintedanib	RTK inhibitor	VEGFR 1-3, PDGFRα, PDGFRβ and FGFR1-3	Boehringer Ingelheim	Phase III
Ramucirumab (IMC-1121B)	MAb	VEGFR-2	Lilly	Phase II/III
Buparlisib	RTK inhibitor	Pan-PI3K	Novartis	Phase I/II