VISUALIZING THE DYNAMICS OF GENETIC PROFILE IN BREAST CANCER TREATMENT: A BETTER WAY TO EXPLAIN WHY A DRUG COULD BE REPURPOSED: A RIVIEW

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SUMMARY

In this work, we further enhance our computational framework for breast cancer drug repurposing by visualizing the prospected dynamic gene expression after the treatment. Practically, the most challenging problem in drug repurposing is to prioritize the list of drugs for further in vivo validation and entering clinical trials. In drug repurposing, the possible candidate drugs could be between fifty and several hundreds, depending on different approaches for candidate selection. In contrast, due to the budget and safety constraints, a repurposing clinical trial usually contain only one or a few drugs. In a prior work, we achieved some successes in solving the prioritization problem. However, we were not able to provide detailed and easy to understand explanation on the prospected dynamic changes of the genetic information. The visualization presented in this work would help achieving this task. The complete framework of computing and visualization helps the doctor to select one repurposed strategy: Targeting ACHE gene in breast cancer for in vivo validation with promising result.

** Keywords: Breast cancer; Drug; Genetics.*

INTRODUCTION

Drug repurposing (also called drug repositioning) has become one of the most active areas in pharmacology since last decade because this approach could significantly reduce the cost and time to invent a new treatment. Before drug repurposing research became active, it was expected to take about 15 years and $0.8 - $1 billion to invent a new drug [1], due to many tests and clinical trials in order to be commercially approved by American Food and Drug Administration (FDA). It is expected that the failure probability during clinical trials is about 91.4% [2]. Briefly, drug repurposing finds new indications for known drugs and compounds [3]. Drug repurposing applies modern computational techniques to digitalize genomic [4], bioinformatics and chemical informatics [5] to offer more systematic evaluation of the chemical compound before entering the laboratory testing and clinical trial steps. In addition, drug repurposing could explore the large set of chemical compounds, which is estimated to be more than 90 million by PubChem (https://pubchem.ncbi.nlm.nih.gov/) statistics, to reduce the cost of synthesizing new compounds. Prominent successful examples for drug repurposing include viagra, avastin, and rituxan [6].

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Practically, in drug repurposing, the researcher solves two problems: Prioritization and explanation. First, in prioritization, given the large number of possible drugs reasonable for repurposing, the researcher needs to estimate which drugs would give the highest chance of success in further in vivo validation. The study at [7] is a typical example of this problem: from the list of thousands drugs approved in the United States, the genetic and pathway analysis, which is among the most well-known method for candidate selection in repurposing, still returns 24 drugs. Therefore, it requires another step of prioritization to select only one or two drugs for validation. Second, after prioritization, the researcher needs to explain why the highly prioritized drugs, which have not been studied for the disease, could possibly help treating the disease. To be more concrete, given that genetic analysis could identify which genes expressing abnormally in the disease, can the drug reverse functionality of these expressing-abnormal genes?. In addition, what is the pathway from the drug's target to these expressing-abnormal genes?.

In this work, we solve the explanation problem given the results from the prior work [8], where we mostly focused on the prioritization problem. By using Gene Terrain technique [9], we can plot the heatmaps of disease-specific gene expression and the expected expression dynamic with the treatment. By comparing these heatmaps, we would be able to estimate which gene expressions would change given the treatment and whether the expressing-abnormal genes would be impacted. Applying the combined approach of [8] and visualization in breast cancer, we help the biologist to select drugs targeting *ACHE* gene, which is originally the strategy to treat the Alzheimer's disease, to be repurposed in treating breast cancer ER-case. The *in vivo* validation shows that targeting *ACHE* gene could inhibit the breast cancer cell line growth, which is a promising result before applying for clinical study.

MATERIALS AND METHODS

1. Reviews from prior study.

In the prior study [8], by modeling the gene expression dynamic in breast cancer and applying system control theory, we suggested 10 drugs promising for breast cancer repurposing. For breast cancer ER+ subtype, the recommended drugs are erbitux, flutamide, medrysone, methylprednisolone, norethindrone, prednisolone, prednisonea and vandetanib. For breast cancer ER - subtype, the recommended drugs are daunorubicin and donepezil. The significant targeting strategy for these drugs could be categorized into:

- Targeting epidermal growth factor receptor (EGFR), which activates several signaling cascades to convert extracellular cues into appropriate cellular responses. Among these signaling pathways are estrogen signaling, in which the receptors ESR1 and ESR2 are well-known for overexpression in breast cancer ER+ [10].

- Targeting acetylcholinesterase (ACHE), which is very popular in the Alzheimer's disease treatment since ACHE participates in neuronal apoptosis [10]. The impact of ACHE in breast cancer, if verify, is very novel.

2. Review: Gene Terrain tool.

Gene Terrain [9], which was initially developed for visualizing gene expression profile, could be further employed to identify the group of disease-specific markers. In gene Terrain, genes having stronger associations would stay closer to each other, laying out on a heat map. In addition, the heat map color is determined by the combinative effect of expression values. Therefore, a group of genes overexpressed or underexpressed together would form a "peak" or a "valley" in the terrain. Therefore, up to this point, the scientist could manually point out the genes inside "peaks" and "Valleys", which are usually much less than the results from GWAS statistical analysis, to identify single marker, as the group of markers. In addition, by comparing the terrains using the expressions of disease, control (non-disease) and treatment subjects, we could find which group of genes express differently among these subjects. The gene Terrain online tool with precise instruction could be found at http://terrainatlas.medeolinx.cn/user/login.

3. Estimating the gene expression with the treatment.

Since the repurposing drugs in section 2a have not been studied in breast cancer, we do not have the expression evidence to use in gene Terrain. Therefore, we estimate the change of gene expressions given the treatment as follow:

$$\mathrm{S}(j,k) = (1-d)c_j + d\sum_{i}^{N} \frac{\mathbf{M}(i,j)\times \mathrm{S}(i,k-1)}{\mathrm{out\_deg}(i)}$$

Here, S: Denotes the vector of estimated gene expression computed iteratively; *N:* Is the total number of genes in the expression profile; *k:* Denotes the k^{th} iteration, *i* and *j:* Denote different nodes; M: Is the matrix of gene-gene associations; out_deg(*i*): Is the gene-degree computed from M; $c_{j:}$ Is the initial value of S(*j*). Damping factor $d = 0.85$ controls how much the new signal S(*j*, *k*) is updated from other nodes in the network. In this work, we only focus on well-known genes appearing in KEGG's breast cancer pathway (https://www.genome.jp/kegg-bin/show_pathway?hsa05224).

RESUTLS

1. Visualizing tamoxifen treatment.

Since Tamoxifen has been approved for treating breast cancer, we examine the tamoxifen visualization to assess the capacity of explanation from the combination of prioritization [8] and gene Terrain [9]. In addition, since we know that tamoxifen may be somewhat ineffective in breast cancer ER-subtype, this case study would demonstrate the "personalized medicine" capacity of the framework. As showed in figure 1, the difference between the ER+ and ER- subtypes include the area of *ESR1-TUBB* genes (1), the area of *BAD-GSK3A* genes (2), and the area of *HPIK2-BAX-ABL1-STK11* genes (3). For the area (1), ESR1 strongly overexpresses in breast cancer ER+ but does not express in breast cancer ER-. Tamoxifen is expected to inhibit ESR1, thus reverses the ER+ subtype but not ER-. Tamoxifen is not expected to have any action in the other areas. Therefore, we can provide an explanation on the difference of Tamoxifen efficacy in treating different subtypes of breast cancer.

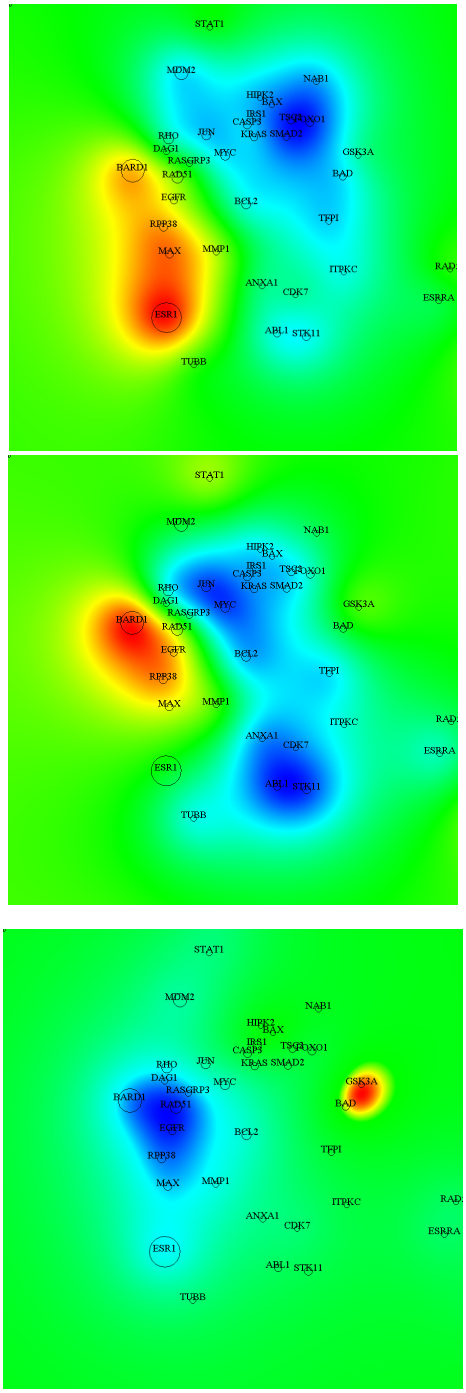


Figure 1: Visualizing tamoxifen treatment. *(Top, left: Breast cancer ER+ gene expression; top, right: Breast cancer ER- gene expression; bottom: Estimated gene expression with tamoxifen treatment)*

2. Visualizing the expectation of targeting EGFR and ACHE treatments.

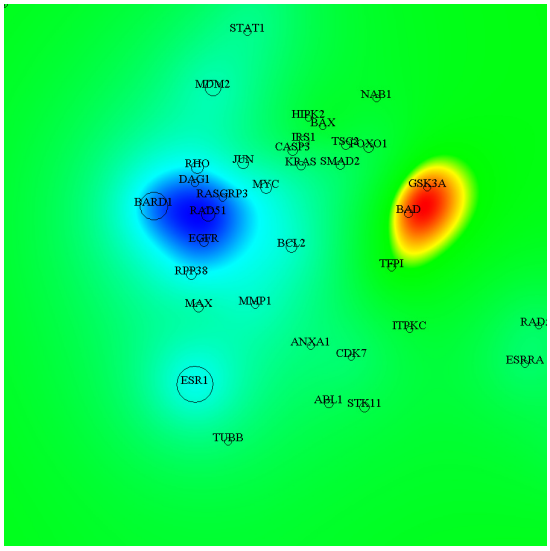
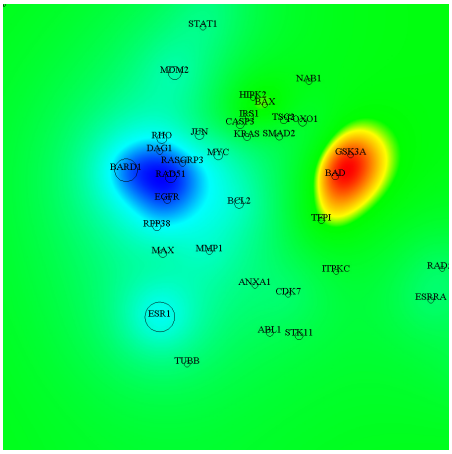


Figure 2: Visualizing targeting EGFR (left) and ACHE (right) treatment.

In figure 2, we show that targeting EGFR and ACHE treatments are expected to have similar gene expression pattern to the tamoxifen treatment. The EGFR and ACHE treatments could lead to the same critical outcome: moderately inhibiting estrogen receptor (ESR1) and strongly inhibiting the group of BARD1-EGFR-RAD51, which strongly overexpress in both breast cancer ER+ and ER- subtypes. We also expect that the EGFR and ACHE strategies could be slightly better than tamoxifen treatment (targeting ESR1) because targeting EGFR and ACHE could activate *BAD* gene (*figure 2*), which is underexpressed in breast cancer ER+ subtype (*figure 1*). Meanwhile, tamoxifen shows now impact on this gene.



3. Further analysis of targeting ACHE.

We focus on targeting ACHE because this strategy has not been explored in breast cancer research, while EGFR has been well-studied in breast cancer (*see figures 1 and 2*). In our *in vitro* validation, the ER+ breast cancer cell line MCF7 and the ER- cell line SKBR3 were treated for 96 hours with escalating of tamoxifen and drug X targeting ACHE. Tamoxifen significantly inhibit both types of breast cancer cell, in which the dosage for the MCF7 cell (IC_{50} = 31.2 ± 4.9 µmol/L) is less than the dosage for SKBR3 cell (IC_{50} = 55.7 ± 4.2 µmol/L). Drug X has the same effect to tamoxifen: it inhibits the MCF7 cell (IC50 = 72.9 ± 5.6 µmol/L) better than the SKBR3 cell (84.6 ± 4.4 µmol/L). However, the dosage needed for drug X is somewhat higher than the dosage needed for tamoxifen. The dosage issue is the major concern before further studying X in clinical trials.

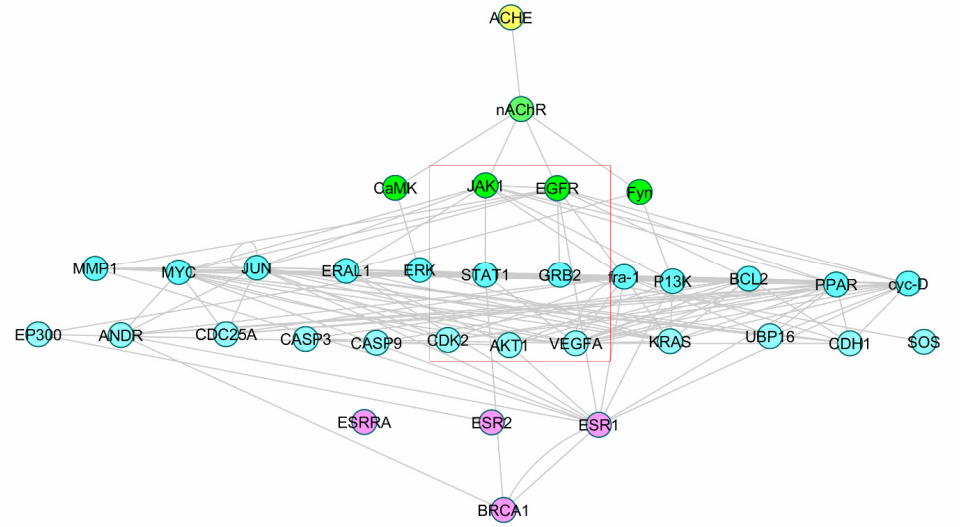


Figure 3: Pathway explaining how targeting ACHE could impact important breast cancer genes.

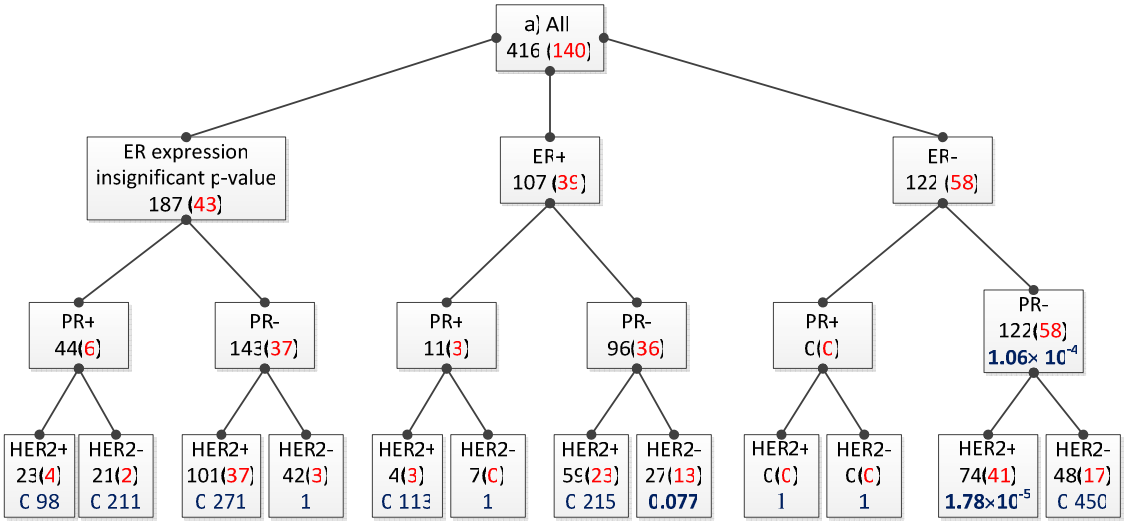


Figure 4: Number of samples in which ACHE overexpresses (red color) according to the expression of PR and HER2 (ERBB2) (black color) in the GSE54002 dataset.

(The p-values of hypergeometric distribution, implying how significantly of observing ACHE overexpressing in specific scenario of ER, PR and HER2 expression, are marked in blue)

To explain why targeting ACHE could impact significant breast cancer gene, we use STRING database (https://string-db.org/) to query the gene-gene regulations and explore the downstream effectors of ACHE. The result showed in figure 3, resembles the patterns of KEGG breast cancer signaling pathway (https://www.genome.jp/kegg- bin/show_pathway?hsa05224). Here, targeting ACHE triggers neuronal nicotinic acetylcholine receptor (nAchR), leading to the activation of the JAK-STAT signaling pathway (in red box). The JAK-STAT signaling pathway triggers the estrogen receptors (ESR1, ESR2), which is, in many cases, the starting point of breast cancer.

In addition, from the GSE54002 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54002), we found that ACHE, strongly expresses in two scenarios: ER+, PR-, HER- (p-value: 0.077), and ER-, PR-, HER2+ (p-value: 1.78×10^{-5} (*figure 4a*). Therefore, targeting ACHE is more likely to treat breast cancer in PR- subtype, or triple negative subtype, in which the common hormone therapy is inefficient.

CONCLUSIONS

In this work, we further investigated the former result at [8] to explain the prospect of breast cancer drug repurposing by using drugs targeting *ACHE* genes. The framework of gene Terrain visualization and pathway analysis allows us to find the potential strategy as above. The ACHE strategy has been partially proven in our in vivo validation. The same framework could be applied to prioritize drug repurposing in other cancer diseases.

However, we have not been able to completely solve the dosage problem.

The experiment shows that although targeting ACHE inhibits the growth of cancer cell similar to the common treatment using tamoxifen, the dosage needed for targeting ACHE is twice more. This dosage may pass the threshold for toxicity in clinical trials. In addition, we show that the dosage may be related to the targeted gene, usually receptor genes, expression. For example, tamoxifen, targeting *ESR1* gene, shows better efficiency in inhibiting breast cancer ER+ cell (having strong ESR1 expression) than inhibiting ER- cell (having weak or moderate expression). Therefore, we suggest that targeting ACHE should only be applied in treating breast cancer with low progesterone (PR) expression. As our result showed, ACHE tends to express stronger when PR level is low.

To conclude, we believe that in Vietnam, drug repurposing should be studied in larger and deeper scale. Not only drug repurposing significantly reduces the cost and time for developing a new treatment but also drug repurposing takes the advantage of systematic techniques and knowledge developed in several decades, organizing in public biochemical databases. In addition, repurposing requires strong mathematical skill, which is usually the major strength of Vietnamese researchers.

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ADVANCES IN THE DIAGNOSIS OF NON-SMALL CELL LUNG CANCER: A REVIEW

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SUMMARY

Lung cancer is the second most commonly diagnosed cancer and remains the leading cause of cancer deaths worldwide. This is often due to lung cancer first presenting at late stages and a lack of curative therapeutic options at these later stages. Radiography and sputum cytology as the screening modalities to early diagnosis of lung cancer but low sensitivity. Advances in the knowledge of the biology of lung cancer have revealed molecular information used for early diagnosis, with an important impact on patients overall survival and quality of life. The recent years, many new techniques are applied in early diagnosis of lung cancer such as: new imaging techniques, advanced bronchoscopy, liquid biopsy. There technologies used and their potential use for non-invasive screening, early diagnosis, prognosis, response to treatment and real time monitoring of the disease, in lung cancer patients.

** Keywords: Lung cancer; Non-small cell lung cancer; New bronchoscopy; Liquid biopsy; Advances in diagnosis.*

INTRODUCTION

Lung cancer is the most common cancer in the world and is the commonest cause of cancer-related death. Audits of patients presenting with lung cancer to hospitals have shown that, at the time of diagnosis, approximately 70% of cases are at an advanced stage (stage IIIB or IV) [4, 5]. Early diagnosis can improve survival. Previous studies showed that using chest radiography and sputum cytology as the screening modalities failed to achieve any significant reduction in lung cancer mortality [4, 10]. In the recent years, many new techniques were applied in early diagnosis of lung cancer such as: new imaging technique and bronchoscopy, liquid biopsies. These techniques can detect early stage asymptomatic lung cancer in high risk peoples, increase the sensitivity of diagnosis and improve survival of lung cancer patients [10, 11]. In this paper we review some new techniques in diagnosis of lung cancer.

LOW DOSE SPIRAL COMPUTERIZED TOMOGRAPHY

The development of low dose spiral computed tomographic (LDCT) imaging has resulted in a resurgence of interest in screening for lung cancer. A LDCT scan is different from a regular computed tomography (CT) scan: the amount of radiation emitted is over five times lower

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than regular CT-scan. LDCT is a more sensitive screening tool for small tumours and can detect early stage asymptomatic lung cancer in a high risk population. The National Lung Cancer Screening Trial demonstrated a reduction in mortality with LDCT annually for 3 years, a median duration of follow-up of 6.5 years. The incidence of lung cancer in the LDCT group was 645 cases per 100,000 person years compared with 572 cases per 100,000 person years in the chest X-ray (CXR) group. LDCT can detect more lung cancers at earlier stages compared with CXR, which results in a significant reduction in mortality. Studies from Japan created excitement in suggesting the viability of LDCT as a tool for early lung cancer detection. The first report was from Kaneko and colleagues, who screened 1,369 high-risk participants with both LDCT and CXR. LDCT detected 15 cases of peripheral lung cancer while 11 of these were missed on chest radiography [2]. Sone and colleagues authored the second report in the literature, with 3,958 participants screened with both LDCT and CXR. Only 4 lung cancers were detected by CXR whereas 19 were seen on LDCT; 84% were stage I at resection. In the United States, Henschke and colleagues with the Early Lung Cancer Action Project: This study enrolled 1,000 high-risk participants and screened with both LDCT and CXR; initial results: A total of 27 prevalence lung cancers were detected by LDCT; only 7 of those were seen by CXR [4, 5]. The ITALUNG study is under way in Italy, where in 3,206 participants have been randomized to LDCT versus no screening. The baseline LDCT was positive (defined as a pulmonary nodule > 5 mm) in 426 (30.3%) of 1,406 subjects. 21% of lung cancers were diagnosed in 20 participants (prevalence 1.5%); 10 (47.6%) were stage I [12].

NEW BRONCHOSCOPY TECHNIQUES

1. Autofluorescence bronchoscopy

Autofluorescence bronchoscopy (AFB), which combines autofluorescence imaging with white light bronchoscopy (WLB), utilizes spectral differences in fluorescence and absorption to distinguish between normal and dysplastic bronchial epithelium. Recent advances include the use of a combination of reflectance and fluorescence [10, 11]. AFB helps early diagnosis and increases the sensitivity of lung cancer diagnosis. The sensitivity of WLB is 9 - 58%, whereas AFB with a sensitivity of 44 - 82%. However, the specificity of AFB is only 46 - 75%, compared with 62 - 95% for WLB. The use of a quantitative score during autofluorescence imaging has been shown to improve specificity.

2. Narrow-band imaging bronchoscopy.

The technique of narrow-band imaging bronchoscopy (NBI) uses a narrow-band filter rather than the conventional, broad, redgreen-blue filter used in standard videoendoscopes. NBI uses three narrow bands: 400 - 430 nm (blue, covering hemoglobin absorption at 410 nm), 420 - 470 nm (blue), and 560 - 590 nm (green). Blue light has a short wavelength, reaches into the bronchial submucosa, and is absorbed by hemoglobin. This technique provides images of microvessels that are more accurate than are those obtained with high-magnification video-endoscopy using broadband RGB technology. The rate of detection of

dysplasia/malignancy obtained with the NBI-WLB combination seems to be higher than that obtained with WLB alone [11, 12]. NBI increases the specificity of bronchoscopy.

3. Endobronchial ultrasound bronchoscopy.

Endobronchial ultrasound bronchoscopy (EBUS) is a technique that uses ultrasound along with bronchoscopy to visualize airway wall and structures adjacent to it. EBUS has been incorporated into routine practice in many centers because of its high diagnostic informative value and low risk. It may replace more invasive methods for staging lung cancer or for evaluating mediastinal lymphadenopathy and lesions in the future. There are two types of EBUS: Radial probe and convex probe EBUS. EBUS with transbronchial needle aspiration (TBNA) has high sensitivity and specificity for identifying malignancy in mediastinal and hilar lymph nodes in patients with lung cancer and also has a high sensitivity for identifying malignancy when used for sampling paratracheal and peribronchial parenchymal lung masses [11]. One of the early studies utilizing EBUS achieved a sensitivity of 94% and specificity of 100% when compared with operative findings. In a prospective comparison of CT, PET, and EBUS in 102 Japanese patients, EBUS had a much higher sensitivity and specificity of 92.3% and 100%, respectively, compared with PET, which was 80% sensitive and 70.1% specific, respectively. A meta-analysis of 11 studies with 1,299 patients who underwent EBUS found a pooled sensitivity and specificity of EBUS of 93% and 100%, respectively. The sensitivity of EBUS increased to 94% in a subgroup of patients selected with imaging compared with only 76% in patients who had no PET or CT selection. The use of EBUS and EUS (esophageal ultrasound) alone resulted in similar sensitivity to surgical staging at 85% (95%CI, 74 - 92%) [12]. The combination strategy also reduced the number of futile thoracotomies by more than half (18% in mediastinoscopy group versus 7% in combination group). The use of PET and EBUS has revolutionized the management of early-stage lung cancer and improved surgical outcomes by optimizing patient selection. The cytology specimens of (EBUS-TBNA) are not only sufficient for histological assessment of lung tumours but also for molecular testing. Reported diagnostic accuracy of EBUS-TBNA in restaging is 95.1% [11].

4. Electromagnetic navigational bronchoscopy.

Electromagnetic navigational bronchoscopy **(**ENB) combines conventional and virtual bronchoscopy to enable the guidance of bronchoscopic instruments to target areas within the peripheral lung parenchyma. ENB consists of a low dose electromagnetic field created around the patient; software that creates a three-dimensional (3D) virtual bronchial tree; a sensor device with navigational capacity that can be located within the magnetic feld; an interface to display the position of the sensor within the yield and input desired target location; an extended working channel (EWC) that enables accurate placement of ancillary bronchoscopic tools, such as brush, biopsy forceps into the target lesion [1]. An open-label, prospective, single-group, controlled clinical study with 15 patients

demonstrated a 69% diagnostic yield. In this study, the majority of these lesions were diagnosed as NSCLC [3]. A recent meta-analysis of 15 trials with a total of 1,033 nodules found a definitive diagnosis was obtained in 64.9% procedures. The sensitivity to detect cancer was 71.1% (95%CI: 64.6 - 76.8%), with a negative predictive value of 52.1% (95%CI: 43.5 - 60.6%) [1].

5. High-magnification videoendoscopy.

The high-magnification Exera endoscopy combines fiberoptic and video-endoscopic technologies to produce images of the bronchial wall at a magnification up to 110 times greater than that obtained with standard video-endoscopy. This enables the visualization of microvascular networks in the bronchial mucosa. Increased vessel density in the bronchial submucosa, which is often presented in squamous dysplasia, might play an early role in cancer pathogenesis [10, 11].

6. Optical coherence tomography.

Optical coherence tomography (OCT) is an optical imaging method that offers microscopic resolution for visualizing structures at or below the tissue surface. it uses near-infrared light (rather than sound waves), which is applied via a small probe inserted into the working channel of a bronchoscopy. Because the velocity of light is far greater than that of sound, the light reflected back from the structures within the tissue cannot be detected electronically, so it is detected with a technique known as low-coherence interferometry. An advantage of this technique is that light waves, unlike sound waves, do not require a coupling medium (liquid or gel), which makes OCT ideal for use in the airways. In addition, OCT creates images of cellular and extracellular structures by analyzing the backscattered light, with a spatial resolution of approximately 3 - 15 µm and a depth penetration of ~ 2 mm, to provide near-histological images of the bronchial wall. Early studies showed that OCT can distinguish dysplasia from metaplasia, hyperplasia, and normal tissue, as well as distinguishing between cancer in situ and invasive cancer.

7. Confocal fluorescence endomicroscopy.

The principle by which confocal microscopy images a thin slide of a sample relies on both the use of a narrow point source on the illumination path and of a small aperture or pinhole on the light detection path. According to this principle, a laser source (the point source) focuses on a single spot in the sample, and the light emitted from this focal point is imaged through the pinhole onto a detector. This results in the rejection of the light coming from depth adjacent to the focal plane region, and therefore of out-of-focus information from the material above and below a very thin plane of focus. Confocal endomicroscopes aim at providing to the clinician ‘‘optical biopsies,’’ that is, in vivo microscopic imaging of living tissue [8]. Proximal bronchial exploration and potential applications for distal lung imaging.

LIQUID BIOPSY

1. Circulating tumor DNA.

Circulating free DNA (cfDNA) can be found dissolved in plasma and serum, at variable amounts. In the case of cancer patients, a fraction of the cfDNA is tumor

derived, and ctDNA represents from less than 0.1% to more than 10% of the total cfDNA. This percentage has been shown to depend on stage, tumor burden, vascularization of the tumor, biological features like apoptotic rate and metastatic potential of the cancer cells. The ctDNA carries the same somatic alterations as the tumor itself and can be used to detect clinically relevant mutations such as those in the epidermal growth factor (*EGFR*) or *KRAS* genes. The European Medicine Agency recommends EGFR testing in liquid biopsies to select patients for tyrosine kinase inhibitor (TKI) therapy [6]. Modifed real-time PCR techniques have been widely used to identify genetic alterations in the cfDNA of cancer patients: Amplifcation-refractory mutation system, Scorpion-ARMS [11], and peptide nucleic acid or locked nucleic acid mutant-enriched PCR.

2. Circulating tumor RNA.

RNA derived from tumor cells (ctRNA) is present in the plasma of cancer patients and can be used for detection of the clinically relevant *ALK*, *ROS1*, and *RET* fusion genes and METΔ14 splicing variant. However, genetic analyses in cfRNA have not been widely used. The recent study has a 5-year experience in detection of EML4-ALK fusion transcripts in plasma cfRNA by retrotranscription PCR (RT-PCR) [7] and, using improved processing and purifcation methods, have demonstrated that the sensitivity of the technique can be signifcantly improved.

3. Tumor educated platelets.

Platelets have been recently demonstrated to sequester tumor RNA by a microvesicle dependent mechanism, and the socalled TEPs can be used as source of tumor RNA for genetic analysis. Platelets can be isolated from blood by simple centrifugation steps, and its RNA content easily purifed and used for the detection of gene fusions and splicing variants. EML4-ALK fusion transcripts in TEP RNA from advanced lung cancer patients with 65% sensitivity and 100% specificity [6, 7]. The disappearance of fusion transcripts in platelets correlates with response to crizotinib treatment. Platelet RNA can also be analyzed by multiplexing techniques, and a recent report has demonstrated the diagnostic potential of this approach. Using mRNA sequencing and surrogate TEP RNA profiles of 283 samples, 228 cancer patients of six different origins were discriminated from 55 healthy individuals with 96% accuracy.

4. Exosomes.

Exosomes are small vesicles present in blood and other body fluids (62 - 64). With a 30 - 100 nm diameter, they are constitutively released through exocytosis by many cells, including tumor cells, in physiological and pathological conditions. Exosomes contain lipids, proteins, mRNA, several types of non-coding RNAs, and double-stranded DNA; and their composition partly reflects that of the parental cells [6]. Exosomes are generally isolated by sucrose gradient ultracentrifugation or immune-bead isolation techniques. Once isolated, exosomes are characterized by transmission electron microscopy, Western blot, FACS, or other methodologies (67). EML4-ALK fusion transcripts have been recently identifed in the exosomal RNA of

NSCLC patients [7]. Some studies indicate that micro RNA (miRNA) analysis of exosomes might be useful for the diagnosis of lung adenocarcinoma (69 - 71) and that particular miRNAs can offer prognostic information in advanced NSCLC.

4. Circulating tumor cells.

Circulating tumor cells (CTCs) are the most widely investigated material in liquid biopsies of cancer patients. In advanced NSCLC patients, CTCs are relatively rare, 1 - 10 per mL against a background of 106 - 107 peripheral blood mononuclear cells. This low abundance poses formidable challenges for the development of robust and sensitive enrichment protocols [6, 7]. Some CTC capture methods are label dependent, based on specific epithelial cell surface markers, such as epithelial cell adhesion molecule (EpCAM) for positive selection or CD45 for negative depletion (the CellSearch® system). In advanced NSCLC, CellSearch® has shown a limited detection effciency, with CTCs detectable in only 20 - 40% of patients. Isolation by size of epithelial tumor cells (ISET®, Rarecells), based on fltration and cytological characterization has shown an increased sensitivity in NSCLC (89 - 92) with an 80% detection rate of CTCs in blood from stage IIIA - IV patients compared with 23% using CellSearch® [7].

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