Cancer Metastasis Rev (2016) 35:129–140
DOI 10.1007/s10555-016-9607-3

Molecular pathology in real time

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Published online: 2 March 2016

Abstract With the development of sophisticated individualized therapeutic approaches, the role of pathology in classification of tumors is enormously increasing. The solely morphological characterization of neoplastic process is no more sufficient for qualified decision on optimal therapeutic approach. Thus, morphologic diagnosis must be supplemented by molecular analysis of the lesion with emphasis on the detection of status of certain markers used as predictive factors for targeted therapy. Both intrinsic and acquired types of intratumor heterogeneity have an impact at various moments of cancer diagnostics and therapy. The primary heterogeneity of neoplastic tissue represents a significant problem in patients, where only limited biopsy samples from the primary tumor are available for diagnosis, such as core needle biopsy specimens in breast cancer, transthoracic or endobronchial biopsies in lung cancer, or endoscopic biopsies in gastric cancer. Detection of predictive markers may be influenced by this heterogeneity, and the marker detection may be falsely negative or (less probably) falsely positive. In addition, as these markers are often detected in the tissue samples from primary tumor, the differences between molecular features of the primary lesion and its metastases may be responsible for failure of systemic therapy in patients with discordant phenotype between primary and metastatic disease. The fact of tumor heterogeneity must be taken into consideration already in establishing pathological diagnosis. One has to be aware that limited biopsy specimen must not always be fully representative of the entire tumor volume. To overcome these limitations, there does not exist one single simple solution. Examination of more tissue (preference of surgical resection specimens over biopsies, whenever possible), use of ultra-sensitive methods able to identify the minute subclones as a source of possible resistance to treatment, and detection of secondary molecular events from the circulating tumor cells or circulating cell-free DNA are potential solutions how to handle this issue.

Keywords Tumor heterogeneity · Evolution · Liquid biopsy · Limitation · Molecular pathology · Targeted therapy · Predictive marker · Resistance

1 Introduction

There are not many areas of modern medicine developing so rapidly as pathology. The fast progress and steadily growing selection of treatment options of cancer in the last decade lead to increasing demand on more precise classification of neoplasms by pathologists. The histopathological diagnosis has to integrate morphological features with molecular findings, such as an expression of different proteins or presence of various genetic alterations. Unfortunately, majority of clinical studies focused on the use of targeted therapy, such as tyrosine kinase inhibitors (TKI), show promising results in prolongation of progression-free survival, but only very few studies were successful in demonstration of the impact of novel therapies on the overall survival. Despite the rapid progress in biomarker testing and drug target discovery, vast majority of patients with metastatic malignancies do—sooner or later—develop resistance to chemotherapy or targeted therapy and show progression and death due to their disease. Development of resistance to systemic treatment thus represents one of the crucial problems of current oncology.

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Multitude of pre-clinical as well as clinical studies has identified various molecular events which are responsible for the phenomenon of resistance. A prominent contributor to therapeutic failure is the intratumor heterogeneity. This can be classified as either primary (intrinsic), which appears early during the carcinogenesis and is present before the systemic therapy is initiated or secondary (acquired). Sequential analysis of tumors has revealed evidence that intratumor heterogeneity temporally evolves during the course of the disease. Acquired heterogeneity therefore results from clonal evolution of the neoplasm, either due to accumulation of spontaneous mutations in genetically instable neoplastic population or due to selection pressure of the systemic treatment [1].

Recent discoveries, however, show that classification of tumor heterogeneity into primary and acquired subtypes is most probably only artificial and therefore arbitrary [2]. At the moment, when malignant neoplasm reaches the clinical detection limits, it is already composed of billions of neoplastic cells which all harbor not only the somatic mutations present in the founder cell, but also additional mutations acquired by generations of daughter cells during the tumor progression, which were passed onto their individual clonal progeny [3]. The acquired heterogeneity is thus frequently mere result of overgrowth of latent minute subclones of cells harboring genetic alterations existing within the tumor even prior to the initiation of therapy; *i.e.*, they have been present in the primary tumor already since the moment of first diagnosis. Only their relative proportion was so low that they could not be detected by standard diagnostic methods [1, 3, 4]. Thus, although most malignancies are of monoclonal origin, during the expansion of the neoplastic population occurring after initial malignant transformation, acquisition of additional somatic mutations results in dramatic increase of intratumor heterogeneity. Such heterogeneity plays a fundamental role in establishing correct diagnosis, in the selection of patients for optimal personalized treatment, and in failure of the treatment due to development of resistance.

The histopathological diagnosis has to be—due to limited options for gaining tissue samples in patients, who cannot undergo operation—frequently rendered from a single tumor biopsy. However, these samples often represent not more than a very small fraction of the entire tumor volume. For instance, it has been demonstrated that a single biopsy of renal cell carcinoma may reveal in average only slightly more than 50 % of all mutations detected in the tumor. Only 34 % of all mutations detected by sequencing of multiple samples from one nephrectomy specimen were present in all regions. This clearly demonstrates that a single biopsy cannot be representative of the mutational landscape of the entire tumor volume [4]. Thus, it is inevitable that discrepancies between the results of various tests performed in both biopsy and subsequent corresponding tumor resection specimen do exist and, in fact, are not rare.

Until recently, intratumor heterogeneity within primary tumors and associated metastatic sites has not been systematically characterized. Novel studies comparing mutational profiles of primary tumors and associated metastatic lesions or local recurrences have provided evidence of intratumor heterogeneity at nucleotide resolution [4]. The more genetically unstable and polymorphic is the initial tumor population, the more significant issue may the heterogeneity of neoplastic population represent.

In the following text, several examples of most common human malignant neoplasms will be used for demonstration of the critical pitfalls in everyday routine diagnostic practice as well as challenges for the future diagnostic approaches.

2 Breast cancer

Molecular classification of breast cancer (BC) and the evaluation of molecular markers such as estrogen receptors (ER), progesterone receptors (PR), HER-2/neu (HER2), and Ki-67 status are considered today as essential parameters for the up-to-date clinical management of BC. Thus, in case of intratumor heterogeneity resulting in potential discordance between primary tumor and metastatic lesion, this may have significant impact in the systemic therapy efficacy. Therefore, it is essential to know what the frequency of discordance of these molecular markers in BC is. There have been nearly 100 papers published in the last 5 years on the topic of concordance/discordance in expression of various prognostic and/or predictive markers between primary BC and its metastases. However, results from different studies are quite heterogeneous and often contradictory, and it is difficult to decide whether these discrepancies reflect true biological difference or just the limited accuracy of testing assays. There are certainly several factors influencing the rate of discordance between the primary tumor and its metastasis; however, two of them are the most crucial.

First, there is a significant difference between synchronous and metachronous metastases [5, 6]. Whilst the synchronous lesions show highly concordant results in testing of both estrogen and PR, discordance in ER between primary tumors and recurrent or metastatic lesions was 12.4 %. There were more positive-to-negative changes (10.1 %) than negative-to-positive changes (2.3 %). Even higher discordance was observed for PR [5]. This illustrates the role of spontaneous development of spatiotemporal heterogeneity with appearance of subclones with different metastatic potential. During the disease progression, certain more aggressive clones metastasizing to specific compartments, such as bone marrow or CNS, may be selected. These secondary populations of neoplastic elements do not necessarily reflect the molecular profile of the primary lesion. In the study published by Bachmann, brain metastases showed more frequently loss of

hormonal receptors (positivity of primary tumor/brain metastases ER—47.6 %/9.0 %; PR—42.9 %/0 %), whereas there is relatively high concordance (>80 %) for HER2 status [7]. Similar situation exists also in HER2 status of BC [6]. HER2 testing is in majority of cases performed in primary BC, and the result serves for treatment selection. The status in primary tumor is assumed to reflect HER2 status of entire tumor load, although HER2 discordance between primary and secondary lesions has been repeatedly described. In the meta-analysis by Houssami [8] covering 26 studies reporting 2520 subjects, pooled HER2 discordance reached 5.5 % (3.6–8.5 %). Interestingly, there was a significant association of type of metastasis with HER2 discordance—higher discordance (11.5 % (6.9–18.6 %)) between primary tumor and distant metastases than with lymph node metastases only (4.1 % (2.4–7.2 %)) was observed. However, some studies showed much higher discordance rate between primary tumor and metachronous distant metastasis, reaching almost 30 % [6]. Thus, it has been recommended that for the decision about treatment of metastatic patients, the evaluation of HER-2 status should be performed in neoplastic tissue from metastatic site, whenever possible.

Second, there is a difference between the treatment-naive *vs*. post-chemotherapy patients; *i.e.*, in treated patients, a therapy-induced clonal selection most probably plays a role [9, 10].

In a study comparing differences in genetic events between primary BCs and asynchronous metastatic/recurrent lesions, Sekido et al. have, using immunohistochemistry, shown that for HER2, p53, ER, and PR, discordance rates between primary and recurrent tumor were 4.5, 2.3, 15.9, and 22.7 %, respectively. By examining HER2 gene amplification and p53 mutation, in cases with discordance between primary tumor and metastasis/recurrence, cell populations present in recurrent tumors were also present in the primary tumors, although they comprised a minor component of the primary tumor. Thus, heterogeneity of the primary tumor apparently contributed to discordance [11].

In a recent meta-analysis published by Aurilio et al. based on 48 selected (mostly retrospective) studies from 1983 to 2011 comparing changes in ER, PR, and/or HER2 status in patients with matched breast primary and recurrent tumors analyzed, a total of 4200, 2739, and 2987 tumors were evaluated for ER, PR, and HER2 discordance, respectively [12]. The heterogeneity between study-specific discordance proportions was high for ER, PR, and HER2. Pooled discordance proportions were 20 % for ER, 33 % for PR, and 8 % for HER2. Pooled proportions of tumors shifting from positive to negative and from negative to positive were 24 and 14 % for ER, 46 and 15 % for PR, and 13 and 5 % for HER2. Thus, majority of studies support the concept of changes in receptor expression during the natural history of BC. This fact may have clinical implications and a possible impact on treatment choice. From the practical point of view, it seems reasonable to perform repeated testing of hormonal receptors and HER2 in patients with late recurrence, as there is quite high chance that the recurrent lesion will have molecular profile different from the primary lesion.

In addition, a possible influence of methodological factors during preanalytical phase should be always taken into account. Typical examples, where failure to meet the standards of preanalytical phase may have critical impact on the results of molecular analysis of BC, are too short (core-cut biopsies) or delayed (large resection specimens with insufficient volume of formalin) fixation or decalcification of the biopsies from bone metastases. These factors should be therefore always considered in the interpretation of immunohistochemistry as well as genetic tests, as stressed by many authors [13–19].

3 Gastric cancer

Gastric carcinoma represents a major health care burden worldwide, mostly in Eastern Europe, South America, and Asia, with more than 900,000 cases diagnosed per year and 700,000 deaths due to the tumor. It is the second leading cause of cancer mortality in the world. Majority of patients present with advanced/metastatic disease; median survival time in such patients is only 8–10 months and a 5-year survival rate only 7 % [20]. Patients undergoing systemic chemotherapy benefit from the treatment in average for 6 months, as most of them develop quite early progression of the disease. Thus, targeted therapy might be of utmost importance for them. Recent studies have demonstrated significant improvement of prognosis in patients with HER2-positive cancer treated with trastuzumab [21]. However, as only tumors with HER2 protein expression and gene amplification may benefit from the treatment, the precise selection of such patients is crucial [20–22].

Unfortunately, gastric carcinoma is an example of malignancy with extremely high heterogeneity, at least regarding HER2 status, and as a consequence, the criteria for HER2 status evaluation have to be different from those used in BC [23]. Unlike in breast tumors, where vast majority of tumors are either HER2 homogenously positive or homogenously negative, in gastric carcinoma, as a rule of thumb, the tumors show highly heterogeneous expression/amplification of HER2 [24–27].

Thus, evaluation of HER2 status from multiple samples from the gastrectomy specimens seems to be more accurate for reliable prediction of anti-HER2 therapy than testing of endoscopic biopsies. Selcukbiricik et al. have shown in a study of 81 patients with radical gastrectomy who were found to have lymph node metastases that the frequency of

concordance in HER2 status, as determined by immunohistochemistry (IHC) or SISH, is high in primary tumors and their corresponding lymph node metastases if testing is performed in the resection specimens [28].

On the other hand, evaluation of HER2 status of gastric cancer from biopsy material only may result in falsely negative or (less frequently) falsely positive results. Proportion of discordance between HER2 statuses estimated from gastric biopsies compared to surgical resection specimens may reach up to 50 % [20]. The most important reason for such high frequency of discrepancies is the spatial heterogeneity of expression with alternation of foci with obvious positivity and complete negativity, as demonstrated by Kimura at al. To overcome the impact of heterogeneity of HER2 protein expression on the result of the test, use of multiple biopsies is strongly recommended [26].

This has been confirmed by Warneke at al. in an experimental study, where they have simulated limitation of HER2 evaluation from biopsy material by random sampling of five tissue cylinders from each gastric resection specimen. The HER2 status in these limited samples has been correlated with the whole tissue sample. They concluded that due to potential sampling error, up to 25 % of patients with HER2 evidently positive gastric cancer would be missed if only biopsy specimens would be available for evaluation. In addition, they showed that limited sampling carries also a minor, but actual risk of a false-positive result. Six cases contained small foci of HER2 overexpressing tumor cell clones which represented <10 % of the entire tumor volume. Sampling of these tumor cell clones would, based on the GC scoring system, result in false-positive test result.

The prognostic importance of these HER2-positive cell clones is not completely clear, as some authors could not show any prognostic significance of HER2 overexpression [24], whereas others have demonstrated a significant inverse correlation between overall survival and HER2 protein overexpression in intestinal-type gastric carcinomas [29]. Nevertheless, the meta-analysis of 15 studies involving 5290 patients showed that HER2 overexpression was an unfavorable prognostic factor for patients with gastric cancer. HER2-positive expression was associated with tumor differentiation, lymph node status, venous invasion, and lymphovascular invasion [30].

In the study of Yan et al., the concordance rate between biopsy material and resection specimens with gastric cancer was only 45.5 %, when as few as 5/11 of HER2-positive resection specimens (IHC 3+ or IHC 2+/FISH amplified) would be identified by IHC 3+ score on matched biopsies. On the other hand, 9/11 cases showed HER2 amplification on matched biopsies with concordance rate 81.8 %. Therefore, it can be recommended that determination of HER2 status by HER2 IHC alone in limited gastric biopsy samples may result in a high false-negative rate and diagnostic accuracy appears to be improved with the additional use of ISH methods [22].

The tumor heterogeneity may be not only a reason for misclassification of HER2 status of the tumor. Even in correctly identified HER2-positive lesion does the level of amplification of HER2 closely correlate with the response to treatment with trastuzumab. Therefore, detailed information about the tumor features is essential for realistic expectations of targeted treatment effect [31].

Due to the inherent limitations of small biopsy samples, whenever possible, the HER2 testing of gastric cancer should be performed in either resection specimens or in samples taken from metastasis. Although the simultaneous determination of HER2 in advanced gastric cancer and matched metastatic lymph nodes is not mandatory, there exists sufficient evidence that the synchronous metastases may have a different HER2 status compared to the primary tumor [27]. Because this discordance may influence the therapeutic management and impact the prognosis, the analysis of HER2 status in synchronous metastasis may provide additional information for such patients and help in optimal selection of patients eligible for targeted therapy with trastuzumab [32]. In patients, where tissue from endoscopic gastric biopsies is the sole source for HER2 status determination, gastroenterologists should be encouraged to carry out extensive sampling and take multiple tumor specimens during the initial gastroscopy, whereas in patients with localized tumors, surgical specimen should always remain a gold standard for HER2 evaluation [25].

4 Colorectal cancer

It is generally accepted today that colorectal cancer (CRC), similarly to malignant neoplasms of any other organ system, is a heterogeneous disease, arising as a result of dysregulation of one of multiple possible pathogenetic pathways [33]. This heterogeneity may have important implications for CRC prognosis and clinical management. Vast majority of CRC tumors develop *via* the traditional pathway through adenoma-carcinoma sequence. In these cases, chromosomal instability, development of p53 mutations, and loss of 18q heterozygosity play the most important role [34]. Another pathway, responsible for the development of carcinomas without preexisting adenoma, is the so-called serrated pathway, where methylation of one of the genes of the mismatch repair system (usually MLH1) resulting in microsatellite instability plays the most important role [35]. The failure of the mismatch repair system can be also caused by the germline mutation of one of the MMR genes and presents as a Lynch syndrome with early onset of CRC [36–39] or, as its clinical variant, Muir-Torre syndrome [40, 41] with high risk of neoplasms in multiple organs (the most frequent ones are sebaceous tumors of the skin, endometrial carcinoma, and urothelial carcinoma)

[42–44]. The phenotype of CRC with microsatellite instability is quite characteristic and includes mucinous differentiation, solid (medullary) circumscribed growth pattern, prominent lymphocytic infiltration, histologic heterogeneity, and right-sided location [45]. Any epidemiological study of CRC risk and prognosis as well as clinical trial must therefore take into account the molecular phenotype of colorectal tumors in patients included in the study [46].

In patients with metastatic CRC, targeted therapy with anti-EGFR monoclonal antibodies (cetuximab, panitumumab) is used for more than a decade. Alterations of the downstream signaling pathway of the EGFR are rather common events in CRC. Despite the original presumption that the expression of EGFR protein (similarly to the expression of HER2 in BC) could be a predictive marker of treatment efficacy, this marker failed in this aspect. Several studies have demonstrated that for the effect of anti-EGFR-targeted therapy, it is the fully functional signaling of EGFR pathway, which must be retained [47, 48], most importantly, the wild-type status of KRAS, NRAS, and BRAF genes [49]. The RAS status is to date the only molecular feature which is from the legal point of view required for decision for choice of targeted treatment in CRC.

As both spatial and temporal heterogeneity represents a significant challenge for treatment targeted on certain signaling pathway, the issue of concordance between primary tumor and the metastatic disease is of crucial importance. Therefore, in several studies, surgical samples from primary and matched metastatic tissues in patients with CRC were evaluated and mutational analysis was performed using a massive parallel sequencing focused on known somatic mutations. Meta-analysis by Baas et al. [50] collected data from 21 studies focused on testing of concordance of KRAS, BRAF, PIK3CA, and loss of PTEN in CRC. The overall reported concordance of KRAS was 93 % (ranging from 76 to 100 %). Overall concordance rates of BRAF status and loss of PTEN testing were 98 and 68 %, respectively. Three studies reported concordance of PIK3CA status, and the concordance range was between 89 and 94 % [50]. The results suggested that pathways between primary tumor and matched liver metastases are mainly conserved, and as a consequence, both primary tumor and tissue from metastatic deposit should be usable for detection of markers predicting the effect of targeted treatment [51, 52]. On the other hand, we get more and more information about the functional status of the tumor cells with unimpaired status of additional signaling molecules, such as PTEN, PI3K, or AKT, and their potential role in the efficacy of the anti-EGFR-targeted therapy. As not all studies reported such high concordance [53] and as the status of these additional downstream molecules is much more often discordant between the primary lesion and the corresponding metastasis [54, 55], it would be probably more reasonable for the future to perform the testing of multiple markers at the same time from the lesion which is targeted for therapy [54]. However, in many patients, the metastatic lesion is not available for the re-biopsy. In those cases, an alternative blood-based test, such as detection of circulating cell-free DNA or circulating tumor cells [56–59], could be used (see below).

5 Non-small cell lung cancer

Non-small cell lung cancer (NSCLC) can be today viewed as a highly heterogeneous group of more or less unrelated neoplasms with variable etiology, molecular pathogenesis, morphology, biology, treatment, and prognosis [60, 61]. Still, only about a decade ago, the basic differentiation into small cell and non-small cell carcinoma subgroups was fully sufficient, as in the group of NSCLC, there were no differences in therapeutic approach between, *e.g.*, adenocarcinoma and squamous cell carcinoma. Thus, any additional subclassification of NSCLC was beyond the therapeutic needs. Only discovery of novel treatment options, which are most efficient in certain subgroups of NSCLC, required division of heterogeneous NSCLC category into adenocarcinoma, squamous cell carcinoma, large cell carcinoma, *etc*. However, even this precise morphological typing is not sufficient anymore. The emerging biologic treatment targeting various molecular signaling pathways is indicated only in patients with neoplasms bearing certain molecular changes, most often one of the so-called drugable driver mutations—typically EGFR-activating mutation or EML4/ALK gene rearrangement. Thus, the classical morphologic diagnosis must be—at least in certain tumor types—accompanied (supplemented) by result(s) of molecular test(s) [62]. According to our current knowledge, individual driver mutations are usually mutually exclusive; therefore, EGFR-positive NSCLC virtually never shows, *e.g.*, ALK rearrangement [60]. Therefore, in addition to morphological classification, tumors can be classified also on the basis of their molecular characteristics. This approach helps the physician in decision what particular drug should be considered for treatment.

Based on these new data, the diagnostic guidelines have been updated in many countries and molecular testing is nowadays an integral part of the diagnostic procedure implemented in complex diagnostics of NSCLC [63]. Routine testing of EGFR mutations for EGFR TKI (gefitinib, erlotinib, afatinib) is performed before the treatment initiation. However, over time (median of 6–12 months), most tumors treated with EGFR TKI develop acquired resistance to the therapy with subsequent progression of the disease. Until recently, there were only limited options for next line of treatment (usually chemotherapy), but since several studies of third generation of TKI showed very promising results, there is increasing need for repeated testing of progressing tumor to specify its molecular profile. Based on available data, there are several different

mechanisms of resistance—T790M mutation in exon 20, MET gene amplification, epithelial-to-mesenchymal transition, EGFR amplification, mutations in the PIK3CA gene, transformation to small cell lung cancer, and HER2 amplification are the most frequent ones [64–70]. In the original reports, preprogression samples lacked T790M, and thus, it was presumed that this abnormality was acquired only after exposure to TKI [71, 72]. However, with the implementation of novel extremely sensitive methods, the resistant subclones harboring these so-called "secondary mutations" can be retrospectively identified even in the initial tumor biopsy or in the circulating tumor cells [73].

Although individual driver mutations (or other genetic abnormalities responsible for carcinogenesis), such as, *e.g.*, HER2 amplification and T790M mutation, are considered to be mutually exclusive, a fraction of patients with EGFR mutation treated by EGFR inhibition develop resistance due to HER2 amplification [69]. These cases support the view that the mutual exclusivity of individual driver mutations is only a virtual phenomenon. One single neoplasm contains numerous cellular subclones harboring several independent molecular events since the early stages and, initially, only one of the mutations is "dominant" whereas the others are suppressed and under the detection limits of current diagnostic methods. Only as a result of either (a) more aggressive potential of one clone or (b) selection pressure induced by treatment blocking/eliminating the most prevalent population, the other clones do get space and conditions for proliferation. This manifests as recurrence of the disease, which now shows different morphology and/or molecular profile [64, 74, 75].

This view can be supported by multiple reports describing various molecular "switches" of originally EGFR mutant tumors progressing on TKI therapy [76, 77]. Turke et al. reported that in EGFR mutant lung cancers, MET amplification activates ERBB3/PI3K/AKT signaling and causes resistance to EGFR kinase inhibitors. Also, MET activation by its ligand, HGF, induces drug resistance. It can be demonstrated by FISH that subpopulations of cells with MET amplification can be identified in the EGFR mutant lung cancers even prior to exposure to targeted treatment by TKI [78].

Third-generation EGFR TKI are highly efficient in patients with cancers harboring the T790M mutation, underlying most first-generation EGFR drug resistance [61, 67]. However, a fraction of patients treated with third-generation TKI, such as rociletinib, develop treatment resistance and show progression. The mechanisms of progression include small cell lung cancer transformation and EGFR amplification. In the study reported by Piotrowska et al. [75], in rociletinib-treated patients, a single pre-rociletinib biopsy showed evidence of molecular heterogeneity as it contained coexisting T790-wild-type and T790M-positive clones. One half of patients progressing on the treatment developed tumors which were completely T790M negative. Thus, the pretreatment fraction of T790M-positive cells was positively affected by rociletinib and T790-wild-type clones were the dominant source of resistance. These findings clearly illustrate the role of tumor heterogeneity, as treatment usually fails if it is targeting just a singular molecular pathway. To improve the outcomes, combination regimens targeting both T790-wild-type and T790M clones could be beneficial [75].

6 Future strategies

Modern diagnostics must aim on the early detection of the evolution of resistant tumor clone(s) resulting in progression of the disease and their early elimination. There are at least three options, how to handle the fact of enormous plasticity of the malignant tumor and heterogeneity of neoplastic population representing a bottleneck in adequate evaluation of tumor phenotype and genotype from limited biopsy samples.

The first one is the introduction of broad and ultra-deep genotyping, which can be efficiently incorporated into routine diagnostic process. The testing in the future should cover broad spectrum of various genetic alterations which are either drugable or contrariwise known to be source of treatment failure. At the same time, it must be sensitive enough to early discover the resistant clone(s) at the moment, when they still represent a minority of the neoplastic population. Only so, the testing may show real clinical utility in influencing treatment decisions and direct patients toward optimal treatment selection. As more targeted therapies are developed, such broad molecular testing will sooner or later become a standard [60, 61]. The multigene panels are already a commercially available option, and some of these panels have been sufficiently validated to show robust enough performance for the implementation into routine diagnostics. However, there is only very limited experience with the use of these tests in the time course for monitoring or surveillance of the treatment efficacy.

The second option, which is currently emerging across different neoplasms and organ systems (melanoma, NSCLC, renal cell carcinoma, colorectal carcinoma), is the totally different approach where there is not targeted any individual molecular pathway, but the treatment helps the immune system to recognize neoplastic elements and destroy them [79]. This immune-oncologic (or immunotherapeutic) approach showed so far quite promising results in multiple clinical studies [80–85]. To understand the benefits and limitation of immune therapeutic approach, one has to analyze the interactions between the neoplastic elements and immune system of the host. For the tumor to become clinically significant, neoplastic population must escape from the immune surveillance of the organism. This is usually achieved by active blockage of the immunity using both suppression of effector populations of immune cells (such as NK lymphocytes) and stimulation of regulatory T cells (T-regs) with inhibiting effect on anti-

tumor immunity [86–88]. The idea of unblocking of immune response and thus helping the organism to fight the cancer by own means has proven to be effective in the clinical trials with new therapeutic antibodies targeted against different molecules regulating the immune response, such as anti-CTLA4 (ipilimumab) or anti-PD1 (*e.g.*, nivolumab or pembrolizumab). Other molecules are emerging, and the results from additional clinical studies should be available quite soon. Unfortunately, these quite promising molecules are efficient in only a minority of patients. At this moment, there are only few examples of well-established clinical or molecular markers which could be used to predict the effect of the treatment in individual patients and the so far published data are rather contradictory. Therefore, there is an enormous need for identification of such biomarkers to make the treatment more efficient.

While there are no putative predictive markers available so far for the ipilimumab therapy, there has been done a lot in the research of predictive markers for anti-PD1 treatment (currently, pembrolizumab and nivolumab). At least in certain tumor types, one of the ligands for PD1 receptor, namely PD-L1 molecule, is being evaluated as a potential predictor of anti-PD1 treatment efficacy. This ligand is expressed in many neoplasms, and as such, it can be detected by IHC in the samples of tumor tissue. Unfortunately, only little is known about the pattern of expression of the molecule—both temporal and spatial heterogeneity exists, and it is not established what is the optimal way for evaluation of expression regarding the distribution of the molecule (center of the tumor *vs*. its periphery), significance of expression in different cell types (positivity in neoplastic cells *vs*. expression by the lymphoid infiltrate), or threshold of positivity [89–91]. In addition, only very little is known about the dynamics of the expression during the course of the disease and how much is the expression influenced by the coincident treatment by other medicaments modifying the immune reaction (such as cytotoxic chemotherapy or use of corticosteroids). Yet, another variable not solved so far is the fact that different clinical studies used different methods (primary antibodies, detection systems, quantification methods, cutoff values) for detection of the PD-L1 expression in the tissue, and so far, there exists no study comparing the pattern and intensity of expression of PD-L1 detected by various primary antibodies and detection kits [92].

Another puzzling issue is the fact that whereas PD-L1 is strongly predictive of treatment effect in certain neoplasms (such as lung non-squamous carcinoma), it has not been predictive in other neoplasms (such as melanoma or squamous cell lung carcinoma). Therefore, although it is not fully clear if the expression of PD-L1 can be used in the latter mentioned tumors, it is highly probable that this marker will be used as a predictor required for starting the treatment in the lung adenocarcinoma. Therefore, histopathological laboratories, which are currently testing predictive markers for other drugs (such as EGFR mutations or EML4/ALK rearrangement), will need to introduce standardized immunohistochemical protocols for this marker. To guarantee constant optimal performance of the testing, all laboratories have to use appropriate internal controls as well as participate in the external quality assurance program.

The third option, which is becoming more and more available, is detection of tumor-specific molecular events in the blood. These so-called "liquid biopsies" might, in the future, supplement the testing of current molecular biomarkers from the tumor tissue. However, there are several limitations of those methods, which so far preclude their routine implementation, at least in the settings where they would replace current standard—detection of mutations from the tumor tissue.

First of all, it should be emphasized that the term liquid biopsy is quite unfortunate, as under this designation are currently covered two fundamentally different approaches—analysis of circulating tumor cells (CTCs) [57, 59, 93] and circulating cell-free DNA (cfDNA). Although there are some recent reports claiming that the detection of mutations from the circulating cfDNA outperforms the CTC approach [94], it is probably too early to expect the final decision regarding superiority of either of these methods. Each of the two strategies has its own pros and cons, and at this moment, it is not possible to predict if one or the other will win the trust of oncologists and prevail in the future [57, 93]. The critical issue in the CTC approach is namely the optimal isolation of sufficient amounts of neoplastic cells from the other blood elements [59, 95]. Interestingly, not all tumor types are shedding the cells into circulation with the same frequency, and thus, it has been demonstrated, *e.g.*, much lower yield of CTCs in colorectal carcinoma compared with breast and prostate cancer, both at baseline and during the course of treatment [59]. In the cfDNA testing field, the main issues are (1) isolation of sufficient amounts of cfDNA from the plasma or serum (even these two sources are substantially different, and the results in one patient may differ depending on the source of cfDNA) [96] and (2) discrimination of cfDNA released from neoplastic cells from the one originating from non-neoplastic elements (such as inflammatory infiltrate, senescent somatic cells, *etc.*). The total amount of cfDNA in blood varies from patient to patient. It correlates with the stage of the disease but differs by orders of magnitude from one neoplasm to the other. The highest amounts of cfDNA have been reported in neuroblastoma, prostatic, ovarian, and colorectal carcinoma (10,000 mutant DNA fragments per 5 ml) whereas in gliomas, medulloblastoma, gastroesophageal, bladder, or renal cell carcinoma, the numbers are dramatically lower (tens to hundreds of fragments per 5 ml). Also, the differences among individual patients with the same diagnosis vary to the same degree [97].

One of the great expectations of cfDNA analysis approach is the circumvention of spatial limitation of the biopsy approach. If we accept the fact of tumor spatial heterogeneity,

it becomes obvious that the small tissue sample cannot represent the entire tumor population (neither primary lesion nor the metastatic load). Circulating DNA should therefore better represent neoplastic tissue load. However, there is lack of information how different tumor subpopulations contribute to the total cfDNA and if this really represents all subclones proportionally or if some of the subclones are more disposed to shed cfDNA molecules than the others.

One of the fields, where concept of cfDNA testing is emerging, is the detection of EGFR mutations in NSCLC [98–100]. The main reason is the fact that patients with NSCLC are usually diagnosed late with advanced disease and thus are not eligible to surgery. In addition, the volume of tumor tissue samples from transthoracic or endobronchial biopsies is mostly very limited. The meta-analysis of 20 studies between 2006 and 2013 was analyzed in total 2012 patients with NSCLC [101]. The pooled specificity of the EGFR mutation detection in the cfDNA was as high as 93.5 %, but the sensitivity reached only 67.4 %. Thus, if only cfDNA testing would be used, one out of three patients with tumors harboring EGFR mutation would be missed and loose the chance to benefit from targeted TKI treatment. This rather limited diagnostic performance thus precludes cfDNA analysis to replace current standard of care—testing of EGFR mutations from the tumor tissue. However, the diagnostic accuracy of different methodological approaches differs quite significantly. While certain methods did not prove sufficient performance and, unlike tissue-based EGFR testing, the cfDNA was not able to stratify correctly patients and thus predict effect of targeted treatment in NSCLC [102], some other methods, like digital droplet PCR, showed similar diagnostic accuracy as tissue-based testing.

Also, as a proof of concept of the use of cfDNA in surveillance of the treatment efficiency, there have been published several papers reporting successful prediction of the failure of the therapy by the monitoring of cfDNA harboring *de novo* appearing resistant mutation, *e.g.*, in colorectal, gastric, breast, or lung cancer [76, 97, 103–109]. Thus, serial monitoring of, *e.g.*, EGFR mutations may allow early detection of resistance mutations [76]. However, studies focused on the question if change of the treatment based solely on the results of resistant clone detection by the cfDNA in the absence of clinical progression is beneficial for the patient (compared to the standard approach when treatment is changed only after the clinically manifest failure of the therapy) are not available so far.

Hence, cfDNA analysis can be, at this moment, used mainly in patients who are not able to undergo repeated biopsy or—for some reason—the tissue obtained from biopsy cannot be used for molecular testing (degraded DNA, insufficient amount of material, relatively low representation of neoplastic cells in the entire cellular population, *etc.*) [98, 110]. Liquid biopsy can in these cases—despite its low sensitivity—add the chance for obtaining of a molecular result, which would be otherwise unavailable [111, 112]. If (or—to be more optimistic—as soon as) the modern technologies help to improve the sensitivity of this diagnostic procedure, one can expect that some genetic alterations will be diagnosed from the blood and the precious tissue specimens will be spared to be used for additional, strictly tissue-based tests, such as immunohistochemical detection of PD-L1, tumor-infiltrating lymphocytes, *etc.*

7 Conclusions

Our current understanding of tumor biology clearly demonstrates that malignant neoplasms are extremely genetically plastic organisms, and as such, they develop—during the course of their life—intratumor heterogeneity. This can be appreciated on morphologic level (various growth patterns within one tumor, areas of heterologous differentiation), but mostly on genetic level. Tumor plasticity and heterogeneity are one of the crucial factors responsible for adaptation of cancer during the chemotherapy and, thus, for development of resistant clones resulting in treatment failure.

The fact of tumor heterogeneity must be taken into consideration already in establishing pathological diagnosis. One has to be aware that limited biopsy specimen must not always be fully representative of the entire tumor volume. This is crucial namely in cases where testing of certain predictive markers from the pre-treatment biopsy is decisive about the selection of targeted therapy. There exists no one single simple solution. Examination of more tissue (preference of surgical resection specimens over biopsies, where possible, testing of metastasis instead of primary lesion), use of ultra-sensitive methods able to identify even the minority subclones, and detection of secondary molecular events from the CTC or cfDNA are potential solutions how to handle this issue.

Acknowledgments This study was supported by the program PRVOUK P37/11, by the project LM2010004, and by grant of the Czech Ministry of Health IGA No. NT14150-3/2013.

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