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Review

Intratumoral heterogeneity and consequences for targeted therapies

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Summary

According to the clonal model and Darwinian evolution, cancer cell evolves through new mutations helping it to proliferate, migrate, invade and metastasize. Recent genetic studies have clearly shown that tumors, when diagnosed, consist of a large number of mutations distributed in different cells. This heterogeneity translates in substantial genetic plasticity enabling cancer cells to adapt to any hostile environment. As targeted therapy focuses only on one pathway or protein, there will always be a cell with the "right" genetic background to survive the treatment and cause tumor relapse. Because today's targeted therapies never took tumor heterogeneity into account, nearly all novel drugs fail to provide patients with a considerable improvement of the survival. However, emerging proteomic studies guided by the idea that Darwinian selection is governed by the phenotype and not genotype, show that heterogeneity at the protein level is much less complex, then it could be expected from genetic studies. This information together with the recent trend to switch from functional to cytotoxic targeting may offer an entirely new strategy to efficiently combat cancer.

Mots clés
Mutation génétique
Rechute tumorale
Résistance aux médicaments
Protéomique
MALDI-TOF

Résumé

Hétérogénéité intratumorale : impact sur le développement de therapies ciblées

Selon le principe de la sélection clonale, dérivée de la théorie de l'évolution énoncée par Darwin, les cellules cancéreuses sont capables d'évoluer et d'acquérir de nouvelles mutations. Ces mutations permettent d'améliorer à la fois leurs aptitudes à proliférer, migrer et envahir les tissus environnants, de même que leur capacité à former des métastases. Des études récentes, dans le domaine de la génétique, démontrent que, lorsqu'elles sont diagnostiquées à temps, les masses tumorales sont constituées d'une vaste population hétérogène de cellules, qui présentent entre elles une variabilité considérable de mutations. Cette hétérogénéité génétique au niveau cellulaire se traduit généralement par une importante capacité des cellules cancéreuses à s'adapter à tout environnement, aussi hostile soit-il, et représente un obstacle majeur au succès des thérapies ciblées, qui, par définition, se concentrent sur une voie de signalisation ou une

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protéine particulière. En effet, étant donné leurs capacités d'adaptation, il subsistera toujours une cellule cancéreuse possédant le profil génétique adéquat pour permettre la résurgence de la tumeur initiale. La plupart des thérapies ciblées développées à l'heure actuelle ne prennent que trop rarement en compte cette hétérogénéité des tumeurs, ce qui explique en partie l'incapacité des nouveaux composés à améliorer considérablement la survie des patients cancéreux. Cependant, une nouvelle vague d'études protéomiques, soutenant l'idée que la théorie Darwinienne dépend principalement du phénotype et non du génotype, tend à prouver que cette hétérogénéité tumorale se révèle, en réalité, bien moins complexe que ce que suggérait la génétique. Cette découverte, associée à l'émergence des thérapies ciblées cytotoxiques au détriment des thérapies fonctionnelles, représente dès lors une stratégie d'avenir et un allié inestimable dans la lutte contre le cancer.

Background

Tumor heterogeneity is one of the most clinically relevant and rapidly evolving fields of basic cancer research. Despite this growing excitement in the fundamental research, clinicians frequently affirm that tumor heterogeneity is a long-standing observation that has little practical impact on the today's management of cancer patients. Although this is unfortunately true for the moment, the recent advances in the field bear the promise to quickly bring a tangible impact. In the current review, we aim to summarize the most important research findings in the field focusing on intratumoral heterogeneity and highlight their direct clinical relevance.

A short historical perspective

The observation that only certain cells from animal tumors can be transplanted to give rise to new tumors originated in the 1930s and was further elaborated in the 1950s when early stem-cell models have been postulated. The latter studies set the track for the modern view of tumor progression, where tumors originate from one clone, with evident chromosomal instability, that undergoes series of selection pressures [1]. These early but visionary concepts were elegantly molded and elaborated by Peter Nowell [2] who postulated the modern clonal model of tumor evolution. This model predicts that genome instability leads to genetic mutations, which in given environment offer selective advantage for a particular clone(s) to grow and expand. The model follows a branched, Darwinian-like evolution, where complementary tracks of evolving clones are possible. In this 1976 paper, Nowell rightly pointed that the resulting heterogeneity (intra- and inter-tumoral) is "discouraging" for any therapeutic consideration and that therapies, regardless how specific they are, will fuel natural selection and tumor resistance. Unfortunately, none of these seminal thoughts were considered when novel cancer therapies were developed in 1980s and 1990s. Recent interest in the tumor heterogeneity was re-discovered at least in part due to failing cancer therapies. As predicted, patients treated with targeted therapies develop resistances and benefit only shortly from these hoped-for cures [3]. Three decades from the Nowell's paper, and with the advent of the genetic technologies able to analyze a single cell [4], we are confirming these early models and the abyss seems broader then ever. It is now for example clear that in one lesion next to divergent evolution a convergent evolution of tumor clones may coexist, and that markers of good and bad prognosis can be detected in different regions of the same tumor [5]. Similarly, mutations thought to be mutually exclusive may occur in the same tumor but in distinct cells [6]. These studies are essential for our understanding of the cancer as disease, but they also rightly raise the question in the clinical community on how they can be translated to the patient.

Lessons from genetic studies

Evolution of species and tumors is similar in many aspects. Both are driven by natural selection based on the phenotype that a cell adopts in a given environment. The potential for adapting to a certain phenotype comes from genes that through mutations give a putative selective advantage. While evolution of species takes naturally thousands of years, cancer cell goes through a similar process in decades. This is possible due to the inherited genomic instability that provokes a plethora of mutations; many of which are disadvantageous but also some drive the tumor progression [7–11]. Persisting genomic instability is promoted by faults in the DNA repair (mutations of key genes like *BRCA1/2*), but also reactive oxygen species (ROS) that arise from abnormal metabolism and offer a perfect tool to perpetually create new DNA insults. Here, it is important to note that increased DNA instability is at the same time a curse and a blessing for the cancer cell. While DNA mutations offer potential for selective advantage over other tumor cells competing for the same resources, they make the cells more prone to apoptosis and mitotic-catastrophe. These cancer cells must override several safety mechanisms (e.g. cell-cycle checkpoints) and use DNA repair mechanisms that are rapid but less fidel. One example of the latter is base-excision DNA repair, which involves PARP and

is especially useful when *BRCA1* and *-2* are mutated (both participate in homologous recombination).

Keeping the previously said in mind, we know today that cancer-initiating clones first acquire few potent founder mutations that deactivate tumor-suppressors (e.g. p53, PTEN) and activate tumor promoters (e.g. *HER2*, *BRAF*, *EGFR*) [12]. As the molecular life of tumor proceeds, more mutations come along and the ability of cancer cell to successfully manage this increased DNA instability is the rate-limiting step in the clonal expansion. Genomic studies assessing tumor needle biopsies show a very consistent picture of a rather stochastic distribution of cells harbouring mutations without a particular pattern [6,13–15]. This has consequences for personalized medicine where biopsies are taken to make diagnostic, prognostic and treatment decisions. These studies clearly show that assessing genetic markers in needle biopsy samples cannot be taken as representative for the entire tumor. As targeted therapies today are ineffective in neo-adjuvant setting, much speaks for the fact that entire lesions need a separate and complete assessment post-surgical removal. Current sampling routines in the clinics are generally not suited to accommodate for such additional tests. As a matter of fact, some malignant lesions are too small to afford additional tests. However, many tumors and particular metastases offer enough material that needs to be well exploited. These practical issues have been recognized by dozen of medical centres around the globe who recently initiated several studies to better conjugate classical pathology routine with research focused on assessing intra- and inter-tumoral heterogeneity. An excellent review on this subject is provided elsewhere [16]. Although an immediate impact for the patient is not expected soon, these studies will critically deepen our understanding of the evolving tumor heterogeneity, especially post-treatment. Recurrence of the metastatic diseases is the principal limitation for curing cancer. Following treatment and subsequent clonal selection, we really do not know much about the personality of the newly evolving tumor.

Much of the today's research in the field of tumor heterogeneity is focused on understanding which mutations have the founder status and which come later in the process [17]. The idea is to pharmacologically target the cells, which bare these important tumor-drivers, and hence, eradicate the relevant clones that promote the tumor growth. Provided we accept this concept as valid, deciding which mutation should be given a founder status is challenging mainly because tumor heterogeneity evolves in time and is different among individuals. For example, EGFR mutations are heterogeneously distributed in at least 1/3 of lung cancer patients, and hence in this subgroup, the EGFR mutation is certainly not a founder event, although in majority of lung cancers this may well be the case [18]. Temporal alteration of heterogeneity is generally more difficult to study because the same lesion needs to be re-biopsied at different times. Some landmark studies have avoided this problem by using genetic models (e.g. based on most-recent common ancestor) and deep sequencing to infer the evolution of the tumor in time [19]. The authors have come across several interesting observations, namely they found that:

- breast tumors at diagnosis bear one major population of cells (more than 50%) that was derived from the same clone;
- long-lived tumor cells tend to accumulate passively DNA mutations for long time without proliferating;
- at the fundament of clonal population, there is a passive long-lived quiescent clone which can expand and regenerate the tumor.

Compelling evidence suggests that metastatic dissemination occurs rather early in the tumor development [20–23]. Circulating tumor cells (CTCs) in patients with no evidence of metastasis (M0) display significantly lower genomic instability compared to CTCs in patients with metastasis (M1) [24–28]. Taken together, these studies have a direct clinical relevance because they show that pathological investigation of the primary tumor in its advanced form is in fact taking a look mainly at cells that have clonally expanded to the point where their mutations became a burden for further tumor progression. Cells fit to metastasize have departed already early in the evolution of the tumor, keeping the fitness to remain plastic and adaptive to the new environment. This is substantiated by recent findings showing that molecular markers for guiding treatment are not necessarily conserved between primary tumor and metastatic lesion [29,30]. To our knowledge, we are unaware of any cancer treatment regiment that takes in consideration this evident difference between the primary tumor and metastatic lesion.

Role of stroma

A paucity of available data underlines the fact that tumor cells need to hijack host cells in order to establish and grow. Failure to successfully manipulate the host tissue into tumor-supportive stroma will result in tumor rejection. In the frame of the current work, we will not review the individual role of fibroblasts, immune cells, adipocytes and endothelial cells in tumor growth and development. This has been done extensively elsewhere [31,32]. The key question that we would like to address here concerns the ability of the tumor cell to differentially manipulate the stroma in order to tease out selective advantages over other tumor clones. A recent eye-opening study on growth factor mediated rescue of receptor tyrosine kinase (RTK) inhibition elegantly shows that for virtually each RTK inhibitor, there is an alternative tyrosine kinase/receptor growth factor couple available, which can rescue the anti-tumor effect [33]. In other words, because RTKs signal downstream through common pathways, blocking one specific growth factor opens doors to those clones that can manage to assure the supply of another. Stroma and in particular cancer associated fibroblast are tuned to produce, if not all, many growth factors. For example, fibroblast-produced hepatocyte growth factor can rescue BRAF inhibition in

melanoma cells with BRAF (V600E) mutation [34], or fibroblast-derived PDGF-C was shown to rescue anti-VEGFA treatment in murine lymphomas [35]. Other components of the stroma, like endothelial cells, can also function as suppliers of factors that can fuel resistance to treatment. Accordingly, endothelial cells in thymus produce IL-6 and TIMP1 when animals are exposed to doxorubicin, creating a favourable environment for tumor cells to survive treatment [36]. Tumor associated macrophages can respond to immune cancer therapies by secreting TNF-alpha which promotes the loss of immunogenic tumor antigens and enhances tumor tolerance [37]. These and other similar studies (extensively reviewed in [38]) clearly show an enormous plasticity of (some) tumor cells to successfully procure necessary factors from the stroma and to escape treatments. Although many therapies are available against stromal components, thus far we were mainly unsuccessful in intercepting and blocking this crosstalk.

Paradigm-shift from emerging proteomic studies

Ample evidence exists to support the idea that genome alterations are required yet sometimes insufficient *per se* to cause cancer. For example, comparison of malignant and benign skin tumors suggests that gene mutations thought to be founder events in cancer can well be present in benign conditions [39,40]. Genetic studies themselves teach us that cells able to metastasize and kill the patient have in fact similar genomes regardless where they eventually settle and grow in the body [41–43]. Both observations point at the enormous plasticity of the most deleterious cancer cells, and hence underline the importance of phenotype over genotype. Epigenetic changes of the DNA are an acknowledged example of a powerful mechanism to achieve a rapid adaptation to the new environment. This fits with Darwin's theory that adaptation is key to survival and that evolution selects a phenotype and not a genotype. However, phenotypic heterogeneity cannot be captured by genetic studies and requires proteomic approaches. Unfortunately, the proteomic studies of intratumoral heterogeneity are still in their infancy and only very limited data are available. Before attempting to summarize the existing work, we would like to make a distinction between peptide/protein Matrix Assisted Laser Disorption Ionization (MALDI)-imaging and classical proteomic studies. The first is a technique that utilizes MALDI based mass-spectrometry to ablate histological sections and produce a 2D spatial map of peptide/protein distribution. The advantage is that the tissue section is analyzed with micrometer resolution allowing for a detailed picture of peptide/protein distribution. The disadvantage is that the technique is currently unable to identify proteins or sequence peptides beyond the most abundant (and inevitably less interesting) species. In sharp contrast to this is the classical proteomics, which usually has no accurate link to the spatial component, but it is routinely able to identify and quantify a considerable portion of the entire proteome. MALDI-imaging has gained a tremendous attention in the past decade with currently well over 1000 papers published, of which a quarter deals with tumors. Inherently to the methodology, MALDI-imaging of a tumor samples is always providing information on the intratumoral heterogeneity. However, this information was so far rarely explored as such and the information depth is generally insufficient for drawing comprehensive conclusions. Recently this trend was somewhat inverted owing to MALDI-imaging studies that explore intra- and inter-tumoral peptide/protein signatures [44]. For example, Balluff et al. [45] have analyzed the intratumoral heterogeneity in gastric and breast cancer and identified tumoral regions bearing signatures relevant to patient survival. Unfortunately, without in-depth proteomics, the study did not identify novel proteins helpful for understanding the underlying biology. Although the authors observed proteome heterogeneity in both gastric and breast cancer, it was striking how concentrated (to some extent organized) are the cells bearing signatures for bad patient outcome. Le Faouder et al. [46] employed MALDI-imaging to analyze hilar and peripheral subtypes of human cholangiocarcinoma. Differential signatures of the two subtypes were highlighted although only few proteins were identified. The authors were able to discern marker proteins found in cancer cells from those found in the stroma. Concerning heterogeneity, the study showed that one protein marker was diffusely distributed in the cancer lesion whereas others were restricted to areas rich in stromal or tumor cells.
If proteomic information on tumor heterogeneity is to be compared to genetic data, then a much deeper and quantitative coverage of the proteome is needed. This is not possible using MALDI-imaging due to inherent limitations of the underlying physical chemistry (e.g. ionization suppression of multiple peptides and drop in sensitivity in the MS/MS mode). Alternatively to the MALDI-imaging approach, there has been newly an increasing effort to include the spatial component in classical proteomic approaches. In one such study, Sugihara et al. [47] have analyzed proteomic heterogeneity in colorectal cancer employing laser-capture micro-dissection and two-dimensional gel-electrophoresis. The authors concluded that the proteomic alterations found in the study were heavily influenced by the microenvironment. The data further evidenced distinct protein signatures for certain biological processes ongoing in the central part of the tumor (e.g. active glucose metabolism), in the ulcer floor (stress response) and in the invasive front (apoptosis). We have recently utilized MALDI-imaging to guide proteomics for analysis of defined regions of interest in human colorectal carcinoma liver metastases (CRC-LM) [48]. Namely, MALDI-imaging allowed the identification of a certain pattern of peptide distribution in CRC-LM, subdividing the lesion in several zones. These zones were then subjected to macro-dissection followed by enrichment and analysis of the accessible portion of

the proteome. The latter group is consisting of extracellular matrix and cell membrane proteins, which are systemically reachable and are particularly relevant for tumor targeting [49]. Using this methodology, we have identified and quantified over 4000 distinct proteins which allowed for:

- a general overview of biological processes and molecular functions and their intratumoral distribution;
- assessing the heterogeneity of biomarkers already used in clinics for therapeutic targeting;
- identification of novel markers for targeted therapies.

The most important information was the finding that proteome heterogeneity in CRC-LM follows a strikingly organized pattern that goes beyond any prediction from existing genomic data. The above-mentioned studies (especially those using MALDI-imaging) critically show that the phenotypic complexity is greatly reduced in comparison to genetic diversity. In our work, we hypothesized that the environment forces a certain phenotype over the cancer cells and that these (owing to their plasticity) will adapt to the given set of conditions. In this respect, we showed that the zonal heterogeneity correlated well with the degree of vascularization in the lesion. The data suggest that oxygen and nutrient availability restrict the degrees of phenotypic freedom. The findings have an immediate clinical impact calling for a more rational drug combination to achieve an optimal coverage of the tumor lesion and hence, limit the possibility for tumor cells to escape and adapt (*figure 1*). Further proteomic studies are needed to mine in-depth different subgroups of proteome bearing the promise for clinical translation. Accessible proteins are an example of such group, while components of RTK signalling may represent another subset worth further analysis.

Consequences for targeted therapies

Targeted therapy is frequently and misleadingly thought of as one entity. A clear distinction between functional therapy and cytotoxic therapy must be made. Both versions of targeted therapy are aiming to target only the tumor/or stroma and not the healthy tissue. However, functional targeted therapy aims at interfering with the function of the relevant protein, whereas cytotoxic-targeted therapy seeks to deliver a toxic drug in a selective fashion. Tumor heterogeneity is a treat to both therapeutic concepts, and clonal selection and evolution theory of tumor development predict that targeted therapy is bound to fail. Inherent to the concept of being very specific, the danger lies in applying the selective pressure and forcing the most plastic cells to adapt and take over. The critical aspect in this process is the dose and the time. Firstly, small compounds interfering functionally with signalling pathways do not accumulate selectively in the tumor [50]. This limitation hampers an efficient escalation of the dose in the tumor lesion, which is required to be high enough to apply the same pressure for all cells having the respective target. Secondly, without a sudden massive damage to the tumor, time is given to the cancer cells to change their personality. Today, post-relapse biopsy is not a standard procedure and tumor heterogeneity limits considerably its meaningfulness. Therefore, the real "personality" of the tumor post-treatment and relapse is elusive and precludes any meaningful strategy to further treat the patient. A true

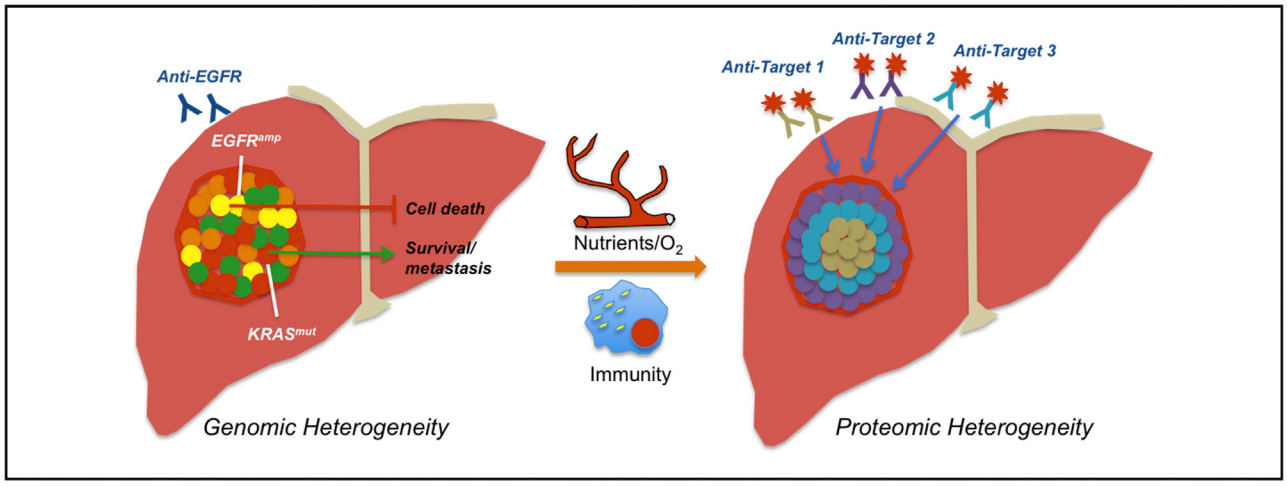

FIGURE 1

Difference between genomic and proteomic levels of tumor heterogeneity

Most of genomic studies report a stochastically distributed genetic heterogeneity. This enables clones with the right mutation (here KRAS) to survive targeted treatments. However, micro-environmental factors, like availability of nutrients, oxygen, growth factors and selection pressure by host immunity impose a certain phenotype that will favour survival. Along these lines, proteomic studies report a much smaller degree of tumor heterogeneity because they study the phenotype and not the genotype of the tumor. This information offers now a possibility to devise combination of cytotoxic-targeted therapies (antibody–drug conjugates) enabling tumor eradication.

alternative is therefore given by the existing toxic targeted therapies. These utilize primarily antibodies as vehicles to deliver toxic payloads [51]. In sharp contrast to small molecules, antibodies accumulate in the lesion, reaching high tumor to blood ratios. The in vivo kinetics can be modulated from hours to days through reformatting the antibodies such that smaller or larger entities can be created. Today, we see a clear trend towards arming naked antibodies, originally developed for functional therapy, into antibody–drug conjugates (ADC). A bonafide example is the newly tested (phase III) trastuzumab–emtansine ADC from Roche, which improved the survival of advanced breast cancer patients by 6 months in comparison to standard care (lapatinib and xeloda) [52]. The trend of arming already clinically approved antibodies will certainly continue. This shift from targeting pure function towards inducing massive damage will certainly profit from new proteomic findings showing that tumor heterogeneity can be targeted in a meaningful fashion. We have in our study [48] named several clinically approved antibodies and quantified their distribution in CRC-LM. Based on this information, it should be conceivable to combine several targeted treatments and test this in reliable preclinical models.

Outlook and future directions

Genetic studies exploring tumor heterogeneity gave us very important hints on tumor biology and shed they light on the emerging resistance to targeted tumor treatment. Based on these information, improvement of functional targeted treatment is expected in the near future, especially after new data become available on the evolving heterogeneity in cancer recurrence. However, targeted cytotoxic therapies depend on protein targets and not on genes. Only proteomic studies exploring the "targetable" proteome and tumor heterogeneity can give better direction on how to meaningfully combine several targeted therapies to achieve better therapeutic effects. Thus far, the proteomic data made available are scarce and frequently not of great use for clinicians. Therefore, it will be essential to conduct further proteomic studies on a well-characterized and statistically significant number of cancer specimens. MALDI-imaging studies, although having a pioneering role in exploring tumor heterogeneity, must be further substantiated with in-depth proteomic analysis. Finally, a considerable effort needs to be placed on extensive validation using clinically relevant methodology (e.g. immunohistochemistry) and large cohorts of patients. Overall, a better integration of genomic and proteomic data will prove powerful enough to both elucidate tumor biology and offer practical guidance to development of newer, smarter, targeted treatments.

Disclosure of interest: the authors declare that they have no conflicts of interest concerning this article.

References

[1] Hauschka TS. The chromosomes in ontogeny and oncogeny. Cancer Res 1961;21:957–74.
[2] Nowell PC. The clonal evolution of tumor cell populations. Science 1976;194(4260):23–8.
[3] Diaz Jr, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012;486(7404):537–40.
[4] Navin N, Kendall J, Troge J, et al. Tumour evolution inferred by single-cell sequencing. Nature 2011;478(7341):90–4.
[5] Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366(10):883–92.
[6] Snuderl M, Fazlollahi L, Le LP, et al. Mosaic amplification of multiple receptor tyrosine kinase genes in glioblastoma. Cancer Cell 2011;20(6):810–7.
[7] Stephens PJ, Tarpey PS, Davies H, et al. The landscape of cancer genes and mutational processes in breast cancer. Nature 2012;486(7403):400–4.
[8] Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. Nature 2013;500(7463):415–21.
[9] Sieber OM, Lipton L, Crabtree M, et al. Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in MYH. N Engl J Med 2003;348(9):791–9.
[10] Pfeifer GP, Denissenko MF, Olivier M, et al. Tobacco smoke carcinogens. DNA damage and p53 mutations in smoking-associated cancers. Oncogene 2002;21(48):7435–51.
[11] Dumaz N, Drougard C, Sarasin A, et al. Specific UV-induced mutation spectrum in the p53 gene of skin tumors from DNA-repair-deficient xeroderma pigmentosum patients. Proc Natl Acad Sci U S A 1993;90:10529–33.
[12] Theillet C. Towards an inventory of oncogenic mutations in cancer. Bull Cancer 2010;97(11):1223–9.
[13] Govindan R, Ding L, Griffith M, et al. Genomic landscape of non-small cell lung cancer in smokers and never-smokers. Cell 2012;150:1121–34.
[14] Shah SP, Roth A, Goya R, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature 2012;486(7403):395–9.
[15] Navin N, Hicks J. Future medical applications of single-cell sequencing in cancer. Genome Med 2011;3(5):31.
[16] Bedard PL, Hansen AR, Ratain MJ, et al. Tumour heterogeneity in the clinic. Nature 2013;501(7467):355–64.
[17] Swanton C. Intratumor heterogeneity: evolution through space and time. Cancer Res 2012;72:4875–82.
[18] Chen ZY, Zhong WZ, Zhang XC, et al. EGFR mutation heterogeneity and the mixed response to EGFR tyrosine kinase inhibitors of lung adenocarcinomas. Oncologist 2012;17:978–85.
[19] Nik-Zainal S, Van Loo P, Wedge DC, et al. The life history of 21 breast cancers. Cell 2012;149:994–1007.
[20] Klein CA. Parallel progression of primary tumours and metastases. Nat Rev Cancer 2009;9:302–12.
[21] Banys M, Gruber I, Krawczyk N, et al. Hematogenous and lymphatic tumor cell dissemination may be detected in patients diagnosed with ductal carcinoma in situ of the breast. Breast Cancer Res Treat 2012;131:801–8.
[22] Hüsemann Y, Geigl JB, Schubert F, et al. Systemic spread is an early step in breast cancer. Cancer Cell 2008;13:58–68.
[23] Sänger N, Effenberger KE, Riethdorf S, et al. Disseminated tumor cells in the bone marrow of patients with ductal carcinoma in situ. Int J Cancer 2011;129:2522–6.
[24] Klein CA, Blankeinstein TJ, Schmidt-Kittler O, et al. Genetic heterogeneity of single

disseminated tumour cells in minimal residual cancer. Lancet 2002;360:683–9.
[25] Schardt JA, Meyer M, Hartmann CH, et al. Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer. Cancer Cell 2005;8:227–39.
[26] Schmidt-Kittler O, Ragg T, Daskalakis A, et al. From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. Proc Natl Acad Sci U S A 2003;100:7737–42.
[27] Murez T, Droupy S, Rebillard X, et al. Circulating tumor cells and advanced prostate cancer. Bull Cancer 2012;99:S4–15.
[28] Weckermann D, Polzer B, Ragg T, et al. Perioperative activation of disseminated tumor cells in bone marrow of patients with prostate cancer. J Clin Oncol 2009;27:1549–56.
[29] Lindström LS, Karisson E, Wilking UM, et al. Clinically used breast cancer markers such as estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 are unstable throughout tumor progression. J Clin Oncol 2012;30:2601–8.
[30] Liedtke C, Broglio K, Moulder S, et al. Prognostic impact of discordance between triple-receptor measurements in primary and recurrent breast cancer. Ann Oncol 2009;20:1953–8.
[31] Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 2012;21:309–22.
[32] Egeblad M, Nakasone ES, Werb Z. Tumors as organs: complex tissues that interface with the entire organism. Dev Cell 2010;18:884–901.
[33] Wilson TR, Fridlyand J, Yan Y, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. Nature 2012;487:505–9.
[34] Straussman R, Morikawa T, Shee K, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature 2012;487:500–4.
[35] Crawford Y, Kasman I, Yu L, et al. PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. Cancer Cell 2009;15:21–34.
[36] Gilbert LA, Hemann MT. DNA damage-mediated induction of a chemoresistant niche. Cell 2010;143:355–66.
[37] Landsberg J, Kohlmeyer J, Renn M, et al. Melanomas resist T-cell therapy through inflammation-induced reversible dedifferentiation. Nature 2012;490:412–6.
[38] Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. Nature 2013;501(7467):346–54.
[39] Hafner C, Lopez-Knowles E, Luis NM, et al. Oncogenic PIK3CA mutations occur in epidermal nevi and seborrheic keratoses with a characteristic mutation pattern. Proc Natl Acad Sci U S A 2007;104:13450–54.
[40] Hafner C, Toll A, Fernandez-Casado A, et al. Multiple oncogenic mutations and clonal relationship in spatially distinct benign human epidermal tumors. Proc Natl Acad Sci U S A 2010;107:20780–85.
[41] Bos PD, Zhang XH, Nadal C, et al. Genes that mediate breast cancer metastasis to the brain. Nature 2009;459:1005–9.
[42] Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 2003;3:537–49.
[43] Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. Nature 2005;436:518–24.
[44] Jones EA, Schmitz N, Waaijer CJ, et al. Imaging mass spectrometry-based molecular histology differentiates microscopically identical and heterogeneous tumors. J Proteome Res 2013;12(4):1847–55.
[45] Balluff B, Frese CK, Maier SK, et al. De novo discovery of phenotypic intra-tumor heterogeneity using imaging mass spectrometry. J Pathol 2015;235(5):3–13.
[46] Le Faouder J, Laouirem S, Alexandrov T, et al. Tumoral heterogeneity of hepatic cholangiocarcinomas revealed by MALDI imaging mass spectrometry. Proteomics 2014;14(7–8):965–72.
[47] Sugihara Y, Taniguchi H, Kushima R, et al. Laser microdissection and two-dimensional difference gel electrophoresis reveal proteomic intra-tumor heterogeneity in colorectal cancer. J Proteomics 2013;78:134–47.
[48] Turtoi A, Blomme A, Debois D, et al. Organized proteomic heterogeneity in colorectal cancer liver metastases and implications for therapies. Hepatology 2014;59(3):924–34.
[49] Turtoi A, De Pauw E, Castronovo V. Innovative proteomics for the discovery of systemically accessible cancer biomarkers suitable for imaging and targeted therapies. Am J Pathol 2011;178(1):12–8.
[50] Krall N, Scheuermann J, Neri D. Small targeted cytotoxics: current state and promises from DNA-encoded chemical libraries. Angew Chem Int Ed Engl 2013;52(5):1384–402.
[51] Smaglo BG, Aldeghaither D, Weiner LM. The development of immunoconjugates for targeted cancer therapy. Nat Rev Clin Oncol 2014;11(11):637–48.
[52] Verma S, Miles D, Gianni L, et al. Trastuzumab emtansine for HER2-positive advanced breast cancer. N Engl J Med 2012;367(19):1783–91.