Pathol. Oncol. Res. (2015) 21:1183–1189
DOI 10.1007/s12253-015-9953-4

RESEARCH

Determination of Tumor Heterogeneity in Colorectal Cancers Using Heterogeneity Tissue Microarrays

Phillip R. Stahl[1] · **Jessica Schnellert**[1] · **Christina Koop**[1] · **Ronald Simon**[1] · **Andreas Marx**[1] · **Jakob R. Izbicki**[2] · **Guido Sauter**[1] · **Alexander Quaas**[1]

Received: 10 December 2014 / Accepted: 14 May 2015 / Published online: 31 May 2015

Abstract Cancer is often heterogeneous both on a morphological and on a genetic level. Though resected tumors are often large, molecular tumor analysis is usually restricted to one tissue block. In this project we introduce a new tool for a high-throughput heterogeneity analysis of colorectal cancer. A heterogeneity tissue microarray (TMA) was manufactured from tissues of 340 patients with colorectal cancer. For this purpose 8 different tissue spots were taken from as many different cancer blocks per patient as possible (at least 4 different blocks). Additional tissue samples from 1 to 4 corresponding lymph node metastases were added from 134 patients. The system was then validated by analysing one parameter each known for minimal (p53) or substantial (HER2) heterogeneity in colorectal cancer. P53 alterations as detected by immunohistochemistry were seen in 174 (51.3 %) of 339 analyzable primary tumors of which 23 (13.2 % of positive cases) showed a heterogeneous distribution pattern. HER2 overexpression was seen in 18 (5.4 %) of 336 evaluable tumors. HER2 amplification occurred in 6 (33.3 %) of the 18 cases with HER2 overexpression. Genomic heterogeneity was more prevalent for HER2 alterations than for p53 alterations. For immunohistochemical expression analysis, 16 of 18 positive cases were heterogeneous (88.9 %) and for amplification 3 of 6 cases (50 %) were heterogeneous. Large section validation revealed, however a considerable fraction of heterogeneous cases were due to technical artifacts. In summary, our data suggest, that heterogeneity TMAs are a powerful tool to rapidly screen for molecular heterogeneity in colorectal cancer.

Keywords Tissue microarrays · Colorectal cancer · Heterogeneity · p53 · HER2

Phillip R. Stahl and Jessica Schnellert contributed equally to this work.

✉ Phillip R. Stahl
p.stahl@uke.de

[1] Institute of Pathology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany

[2] General, Visceral and Thoracic Surgery Department, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany

Introduction

Colorectal cancer is the third most common malignancy worldwide [1] with surgery representing the only curative treatment. Treatment is often complemented by chemotherapy [2, 3]. As in other tumors, colorectal cancer carcinogenesis is supposed to be a multistep process with accumulation of several gene alterations.

Mutation of the p53 gene is believed to trigger the transition from adenoma to invasive cancer in a large fraction of colorectal cancers [4]. P53, located on chromosome 17, is a tumor suppressor gene and plays a crucial role in tumor suppression by mediating apoptosis, inducing cellular senescence, controlling cell cycle progression, and possibly also further mechanisms [5, 6]. P53 mutations can be seen in about 40–50 % of colorectal cancers and tend to be associated with a poorer outcome [7–10].

HER2 is involved in the regulation of cell proliferation and differentiation [11] and serves as a therapeutic target in HER2-positive breast and gastric cancer [12, 13]. However, many other cancer types are known to show increased HER2 expression levels [14–16] and it is more and more getting evident that anti-HER2 therapy might also be effective in other tumor entities such as HER2 positive colorectal cancer [17, 18].

Treatment with trastuzumab and other targeted drugs requires a previous molecular analysis of tumor tissue. The

molecular tumor status is generally determined only on a small fraction of the primary tumor. Under these circumstances it is not possible to make a reliable statement with respect to the molecular status of the entire cancer mass. Accordingly molecular cancer heterogeneity may represent one of the main reasons for limited efficiacy of standard chemotherapy and targeted drugs. Several studies indicate that intratumoral heterogeneity plays an important role in drug resistance especially in tumor entities for which a targeted therapy is approved. These are for instance chronic myleogenous leukemia (Imatinib) and melanoma (Vemurafenib) [19]. Moreover, molecular cancer heterogeneity may also spoil the analysis of genes or gene products serving as diagnostic or prognostic markers.

In order to evaluate molecular cancer heterogeneity, we have recently introduced a novel tissue microarray (TMA) platform, which we termed "heterogeneity TMA" [20]. Such heterogeneity TMAs contain multiple tissue cores per tumor distributed across the entire tumor mass and taken from all available cancer containing tissue blocks. To validate this concept for the analysis of colorectal cancers we analysed 340 patients for p53 and HER2 heterogeneity. The data demonstrate highly different levels of heterogeneity for these biomarkers and demonstrate the power of analysing heterogeneity by TMAs in a high-throughput manner.

Material and Methods

Patients/TMA Manufacturing A heterogeneity TMA was constructed for this study. Quantitative screening of several hundred colorectal cancers for tumor tissue revealed 340 cases with at least 4 blocks of paraffin embedded tumor tissue. For each patient 8 tissue cores from different areas of the primary tumor were punched out and transferred in a TMA recipient block. In cases where less than 8 tumor containing tissue blocks were available, additional cores were taken from the largest tumor blocks. In these cases samples were taken from tumor areas that were as distant from each other as possible. Furthermore, tissue cores (one each) from 1 to 4 corresponding lymph node metastases of 134 patients were added. TMA construction was as described [21]. In brief, tissue cylinders with a diameter of 0.6 mm each were punched from selected tumor "donor" tissue blocks using a home made semiautomated precision instrument and brought into empty recipient paraffin blocks. Four μm sections of the resulting TMA blocks were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, NJ). Consecutive sections were used for fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC).

Immunohistochemistry (IHC) For HER2 IHC the HercepTestTM (DAKO) was used according to the protocol of the manufacturer. Immunostaining was scored by one pathologist following a 4-step scale (0, 1+, 2+, 3+). Scores 2+ and 3+ were counted as "HER2 positive", 0 and 1+ as "HER2 negative". For p53 IHC following antibody was used: DO-7 (DAKO, Cat. No: IR616, monoclonal mouse, ready to use, pH9). For each tissue sample, the percentage of p53 positive tumor cells was estimated and the staining intensity was recorded semiquantitatively as 1+, 2+ or 3+. For statistical analysis, the staining results were categorized into four groups. Tumors without any staining were considered negative. Tumors with 1+ staining in ≤70 % and tumors with 2+ staining in ≤30 % of cells were considered weakly positive. Tumors with 1+ staining in >70 %, tumors with 2+ staining in >30 % but ≤70 % and tumors with 3+ staining in <30 % of cells were considered moderately positive. Tumors with 2+ staining in >70 % and tumors with 3+ staining in ≥30 % of cells were considered strongly positive.

Fluorescence In Situ Hybridization (FISH) Four μm sections were used for two-color FISH. For proteolytic slide pretreatment, a commercial kit was utilized (paraffin pretreatment reagent kit; Vysis-Abbott, Chicago, IL, USA). A SpectrumOrange-labeled HER2-probe was used together with a SpectrumGreen-labeled CEP 17 probe (PathVysion; Vysis-Abbott) for HER2 analysis. Before hybridization, sections were deparaffinized, air-dried, dehydrated and then denatured at 72 °C for 5 min in a ThermoBrite (Abbott). After overnight hybridization at 37 °C in a ThermoBrite, slides were washed and counterstained with 10 μl DAPI I (Abbott). For each tumor, the predominant gene and centromere copy numbers were estimated. Based on these numbers, a tumor was considered amplified if the HER2 / centromere 17 ratio was ≥2.0 or if tight HER2 gene clusters were present. Amplifications were considered "high level" if the HER2 / centromere 17 ratio was ≥4.0 or if tight HER2 gene clusters were present.

Large Section Validation

A large section validation of all (n=57) available tumor tissue blocks from 14 p53 positive patients was performed.

Results

P53 Analysis At least 3 interpretable primary tumor tissue samples were available from 339 patients. Numbers were assigned according to the staining results of each individual tissue spot (negative="0", weak="1", moderate="2", strong="3"). Patients/tumors were then categorized as follows:

1. A total of 92 (27.1 %) tumors showed a staining result of "**0**" in all analyzable tumor spots representing a group of totally p53 negative tumors.

2. A total of 73 (21.5 %) tumors showed a staining result of "**0**" and "**1**" in at least one analyzable tumor spot, respectively. These tumors were also considered p53 negative.
3. A total of 151 (44.5 %) tumors showed a staining result of "**1**" or "**2**" or "**3**" in all analyzable tumor spots representing a group of homogeneous p53 positive tumors.
4. A total of 23 (6.8 %) tumors showed a staining result of "**0**" and "**2**" or "**3**" in at least one analyzable tumor spot respectively representing a group of heterogeneous p53 positive tumors.

A schematic representation is given of the results for the groups "2" and "4" in Fig. 1.

Large Section Validation Large section validation of a total of 57 available tumor containing tissue blocks from 14 randomly selected patients of the p53 heterogeneous group confirmed a heterogeneous p53 distribution pattern in 8 patients. In these patients a sharp demarcation was seen between p53 positive and negative cancer areas.

The remaining 6 validated tumors showed either homogeneous p53 immunostaining ($n=5$) or complete absence of p53 staining ($n=1$) across the entire tumor mass on large sections. In the five homogeneously p53 positive cases, focal IHC failure had to be assumed for our TMA analysis. Retrospective evaluation of the one case converted to homogeneous p53 negativity revealed that in this case only 1 of 7 analyzable TMA spots was recorded as "**2**" whereas all other tumor spots were completely negative.

P53 in Metastases Interpretable p53 results for 1–4 lymph node metastases were available for 120 patients. In all these cases IHC results were also available for corresponding primary tumors from at least 3 tissue spots. Concordant p53 staining results between metastases and corresponding primary tumors were seen in 118 (98.3 %) of 120 cases. Large section validation revealed that the discordant results in two cases were due to a "sampling error" and IHC artifacts caused by inhomogeneous tissue fixation, respectively.

HER2 IHC A total of 336 patients had at least 4 interpretable tissue samples. A total of 18 (5.4 %) patients showed a positive (at least **2**+) HER2 immunostaining in at least one tumor spot. Only 2 cases showed homogeneous HER2 expression with a staining result of "**2+**" and/or "**3**+" in all analyzable tumor spots. In 16 (88.6 %) of 18 cases HER2 immunohistochemistry suggested a heterogeneous distribution pattern with a staining result of "**0**" and "**2+**" or "**3+**" in at least 1 analyzable tumor spot, respectively (Table 1).

HER2 FISH All IHC positive tumors were validated by HER2 FISH on the TMA. FISH analysis revealed HER2 amplification in 6 (33.3 %) of the 18 IHC positive cases. Three cases (including the two cases with homogeneous HER2 IHC positivity) showed homogeneous HER2 amplification (2 with high-level amplifications, 1 with a low-level amplification). The third homogeneously amplified cancer had only one corresponding TMA spot with a **2+** IHC, while all others were negative (**0**). Large section validation of this cancer confirmed homogeneous amplification and revealed a continuous decrease of immunoreactivity along the slide suggesting a preanalytical problem affecting IHC. Three additional cases showed a heterogeneous HER2 amplification status (all of them with low-level amplifications) (Table 1).

HER2 in Metastases Interpretable HER2 IHC data were available from 1 to 4 lymph node metastases of 124 patients for which HER2 IHC results were also available from the corresponding primary tumors (at least 4 tissue spots). There was a good but not perfect association between primary tumors and metastases. Concordant HER2 staining results between metastases and corresponding primary tumors were seen in 120 (96.8 %) of 124 cases.

Interpretable data were available for 10 patients with at least one HER2 positive spot (primary tumor or metastasis). All these cases had interpretable tumor tissue on at least 6 spots.

Four (40 %) cases showed discordant results. Large section validation revealed that the discordant results were either due to a "sampling error" or IHC artifacts caused by inhomogeneous tissue fixation, respectively.

Discussion

Tumor heterogeneity is a critical issue and a major limitation for molecular diagnostics and targeted cancer therapy. Diagnostic accuracy of a molecular assay may be limited if the analyzed biomarker is only present in a fraction of a tumor. Absence of a drug target structure in a cancer subpopulation of a patient tested "positive" for a specific drug target may cause drug resistance after outgrowth of the target-negative population under therapy. If tumor heterogeneity is analyzed and quantified this is often only based on the analysis of one slide/block per tumor/patient. However, one tissue section may not completely represent the biology of a large cancer.

As the analysis of multiple large tissue sections for the evaluation of large tumor cohorts is tedious and costly, a new tool for studying molecular cancer heterogeneity was manufactured for this study. The TMA analysis of eight different tissue samples taken from 4 to 8 different tumor-containing tissue blocks enables a rapid but comprehensive molecular analysis of molecular features in a large series of tumors. The heterogeneity TMA concept introduced in this project differs markedly from previous attempts to increase

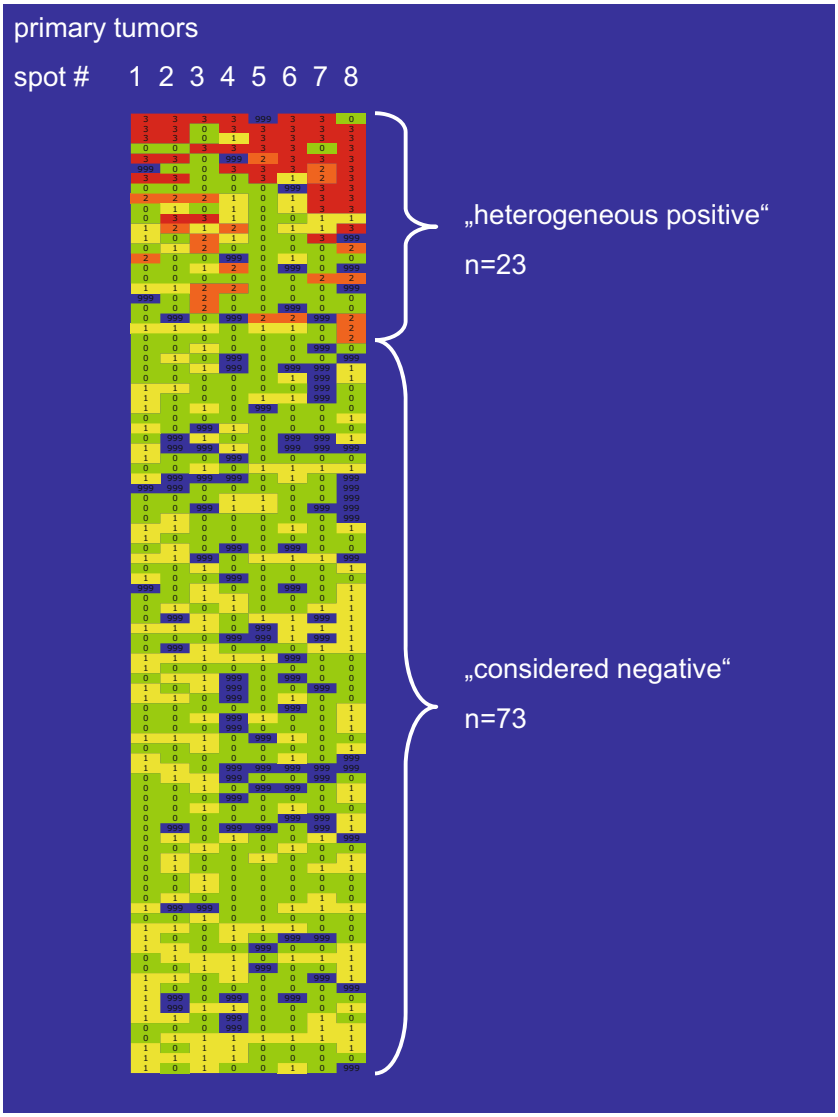

Fig. 1 Schematic representation of the results for the groups “2” and “4” of our p53 analysis. The different *colours* represent the staining results of each individual tissue spot (*blue*=not analyzable, *green*=negative, *yellow*=weak, *orange*=moderate, *red*=strong)

the representativity of TMAs by sampling multiple cores from just one tumor block [22–24].

Our heterogeneity TMA analysis for p53 revealed 51.3 % positive cases. This fraction is well in the range of many previous studies [9, 10]. Only 23 (6.8 % of the entire cohort) tumors showed a heterogeneous p53 distribution pattern, suggesting a highly homogeneous distribution of p53 alterations in colon cancer. An extensive large section validation of all available tumor blocks from 14 tumors of this group confirmed heterogeneity in only 8 cases. In the remaining 6 cases few false results – due to technical artifacts - had led to the erroneous assumption of heterogeneity. This observation illustrated the critical need for technical perfection in heterogeneity studies. In such a project, every technical error will lead to a deviation from normality in homogeneous cancers and thus lead to “false” heterogeneity. It is obvious, that experimental errors must always be expected in studies using immunohistochemistry on clinical specimens undergoing normal variability of fixation such as for example duration of fixation, ratio of fixative per tissue quantity, and temperature during fixation [25].

Only a few earlier studies had analyzed heterogeneity of p53 alterations in colorectal cancers by immunohistochemistry [9, 10, 26]. The lower rate of heterogeneous findings observed in these studies is likely to be due to the fact, that only one large section/tissue block was analysed in these studies. The low rate of heterogeneity of p53 alterations further supports the well established assumption of p53 mutations representing an early event in the development of a large fraction of colorectal cancers [4].

Our heterogeneity TMA evaluation of 336 analyzable tumors revealed 18 (5.4 %) HER2 IHC positive and 6 (1.8 %) HER2 amplified cases. This frequency is well in the range of several previous studies [27–29]. However, several other groups had indicated much higher rates of HER2 expression [30, 31]. These inconsistent findings are probably due to the

Table 1 HER2: FISH vs IHC

Results										
	Spot#	1	2	3	4	5	6	7	8	Rating
Case										
1	IHC	3	3	3	3	3	3	3	3	Hom
	FISH	$\frac{20-50}{2-4}$	$\frac{20-50}{2-4}$	$\frac{20-50}{2-4}$	$\frac{20-50}{2-4}$	$\frac{20-50}{2-4}$	$\frac{20-50}{2-4}$	$\frac{20-50}{2-4}$	$\frac{20-50}{2-4}$	Hom
2	IHC	2	2	3	3	2	2	na	na	Hom
	FISH	$\frac{4-6}{2}$	$\frac{4-12}{2}$	$\frac{6-12}{2-4}$	$\frac{4-8}{2-3}$	$\frac{2-6}{2-3}$	$\frac{4-6}{2-3}$	na	na	Hom
3	IHC	na	na	0	na	0	2	0	0	Het
	FISH	na	na	$\frac{10-20}{2}$	na	$\frac{8-15}{2-3}$	$\frac{10-20}{2-3}$	$\frac{15-25}{2-4}$	$\frac{15-30}{2-4}$	Hom
4	IHC	0	na	na	2	0	na	2	0	Het
	FISH		na	na	na	$\frac{2}{2}$	$\frac{2}{2}$	$\frac{4-10}{2-5}$	na	Het
5	IHC	3	3	0	0	3	na	na	3	Het
	FISH	$\frac{6-12}{2}$	$\frac{6-15}{2-3}$	$\frac{2}{2}$	$\frac{2}{2}$	$\frac{6-12}{2-4}$	$\frac{4-8}{2-3}$	$\frac{4-8}{2}$	$\frac{8-15}{2-4}$	Het
6	IHC	2	0	0	na	na	0	1	1	Het
	FISH	$\frac{2}{2}$	$\frac{4-6}{2}$	$\frac{4-6}{2}$	$\frac{4-6}{1-3}$	na	$\frac{2}{2}$	$\frac{3-6}{2}$	$\frac{4-6}{2-3}$	Het
7	IHC	0	0	2	0	0	0	na	0	Het
	FISH	$\frac{1-2}{1-2}$	$\frac{1-2}{1-2}$	$\frac{1-2}{1-2}$	na	na	na	$\frac{1-2}{1-2}$	na	No amp
8	IHC	0	0	0	0	2	0	2	0	Het
	FISH	$\frac{2}{2}$	$\frac{2-3}{2-3}$	$\frac{2-4}{2-4}$	$\frac{2}{2}$	$\frac{4-6}{3-5}$	$\frac{2}{2}$	$\frac{2-4}{2-4}$	na	No amp
9	IHC	0	0	2	0	0	0	0	na	Het
	FISH	$\frac{2-3}{2-3}$	$\frac{2}{2}$	na	na	$\frac{2-3}{2-3}$	$\frac{2-5}{2-4}$	$\frac{2-3}{2-3}$	$\frac{2-4}{2-4}$	No amp
10	IHC	2	2	na	0	na	na	na	0	Het
	FISH					na	na	$\frac{2-3}{2-3}$	$\frac{2-4}{2-4}$	No amp
11	IHC	2	3	0	na	0	3	2	1	Het
	FISH		$\frac{2}{2}$		na	$\frac{2}{2}$	$\frac{2-5}{2-4}$	$\frac{2-3}{2-4}$	$\frac{2-3}{2-3}$	No amp
12	IHC	2	1	0	na	0	0	2	0	Het
	FISH		na	$\frac{2}{2}$	$\frac{2}{2}$	$\frac{2-3}{2-3}$	$\frac{2-3}{2-3}$	$\frac{2-3}{2-3}$	$\frac{2-4}{2-4}$	No amp
13	IHC	2	1	0	0	1	0	0	0	Het
	FISH	$\frac{2}{2}$								No amp
14	IHC	0	2	2	0	0	0	2	0	Het
	FISH	$\frac{2}{2}$	na	$\frac{2}{2}$		na	na	na	na	No amp
15	IHC	0	0	1	0	0	2	0	0	Het
	FISH	$\frac{2}{2}$	$\frac{2}{2}$	$\frac{2-3}{2-3}$	$\frac{2-3}{2-3}$	$\frac{2-3}{2-3}$	$\frac{2-3}{2-3}$	$\frac{2}{2}$	$\frac{2-3}{2-4}$	No amp
16	IHC	na	0	0	0	na	2	0	0	Het
	FISH	na	$\frac{2-5}{2-4}$	$\frac{2-4}{2-4}$	$\frac{2-4}{2-3}$	na	$\frac{2}{2}$	$\frac{2-4}{2-3}$	$\frac{3-6}{2-4}$	No amp
17	IHC	0	3	3	0	0	0	0	0	Het
	FISH	$\frac{2}{2}$	$\frac{2}{2}$	$\frac{2}{2-3}$	$\frac{2-3}{2-3}$	$\frac{2}{2}$	$\frac{2}{2}$	$\frac{1-2}{2}$	$\frac{2}{2-3}$	No amp
18	IHC	0	0	0	0	3	3	1	0	Het
	FISH	$\frac{2-4}{2-4}$	$\frac{2-4}{2-4}$	$\frac{2-3}{2-4}$	$\frac{2-4}{2-4}$	$\frac{2-3}{2-3}$	$\frac{2-4}{2-4}$	$\frac{2-3}{2-3}$	na	No amp

Abbreviations: *amp* amplification, *Het* heterogeneous, *Hom* homogeneous, *na* not analyzable

different approaches of performing IHC analysis, including the use of different antibodies and variations in staining interpretation [28, 32]. The markedly higher rate of HER2 positivity found by IHC than by FISH in our study is consistent with the known risk of HER2 IHC for false positivity, especially in poorly fixed tissues [25].

Our result of HER2 heterogeneity in 3 of 6 cancers with unequivocal HER2 gene amplification is consistent with earlier data from our group describing HER2 heterogeneity in 3 of 4 cases found amplified on a large-scale colon cancer TMA [29].

Several cases of successful trastuzumab therapy outside of established indications (breast and gastric cancer) are described in the literature [33–36]. In another study we reported the successful treatment of a 69 year-old colon cancer patient with trastuzumab [17]. These data suggest that further attempts applying trastuzumab in HER2 positive colon cancer and other entities are warranted, in particular in case of homogeneous HER2 status.

Conclusion

In this study a novel "heterogeneity TMA" approach was used to show different heterogeneity levels for two important molecular changes in colorectal cancer. The low degree of heterogeneity found for p53 alterations supports the notion of p53 mutations representing an early event in the development of many colorectal cancers. The much higher degree of heterogeneity observed for HER2 amplification is consistent with a much later role of HER2 activation in colorectal carcinogenesis. Overall, our results support our heterogeneity TMAs as a powerful tool for rapidly analyzing the distribution pattern of genetic alterations in primary tumor subpopulations as well as in corresponding metastases.

Conflicts of Interest The authors have no conflict of interest to disclose.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61:69–90
2. Lombardi L, Morelli F, Cinieri S et al (2010) Adjuvant colon cancer chemotherapy: where we are and where we'll go. Cancer Treat Rev 36:S34–S41
3. Van Cutsem E, Köhne CH, Hitre E et al (2009) Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med 360:1408–1417
4. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. Cell 61:759–767
5. Vousden KH, Prives C (2009) Blinded by the light: the growing complexity of p53. Cell 137:413–431
6. Levine AJ, Oren M (2009) The first 30 years of p53: growing ever more complex. Nat Rev Cancer 9:749–758
7. Russo A, Bazan V, Iacopetta B et al (2005) The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. J Clin Oncol 23:7518–7528
8. Munro AJ, Lain S, Lane DP (2005) P53 abnormalities and outcomes in colorectal cancer: a systematic review. Br J Cancer 92: 434–444
9. Jourdan F, Sebbagh N, Comperat E et al (2003) Tissue microarray technology: validation in colorectal carcinoma and analysis of p53, hMLH1, and hMSH2 immunohistochemical expression. Virchows Arch 443:115–121
10. Scott N, Sagar P, Stewart J, Blair GE, Dixon MF, Quirke P (1991) p53 in colorectal cancer: clinicopathological correlation and prognostic significance. Br J Cancer 63:317–319
11. Rubin I, Yarden Y (2001) The basic biology of HER2. Ann Oncol 12:S3–S8
12. Slamon DJ, Leyland-Jones B, Shak S et al (2001) Use of chemotherapy and a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783–792
13. Bang YJ, Van Cutsem E, Feyereislova A et al (2010) Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet 376:687–697, Epub 2010 Aug 19. Erratum in: Lancet. Oct 16;376(9749):1302
14. Tapia C, Glatz K, Novotny H et al (2007) Close association between HER-2 amplification and overexpression in human tumors of non-breast origin. Mod Pathol 20:192–198
15. Hu Y, Bandla S, Godfrey TE et al (2011) HER2 amplification, overexpression and score criteria in esophageal adenocarcinoma. Mod Pathol 24:899–907
16. Williams MD, Roberts DB, Kies MS, Mao L, Weber RS, El-Naggar AK (2010) Genetic and expression analysis of HER-2 and EGFR genes in salivary duct carcinoma: empirical and therapeutic significance. Clin Cancer Res 16:2266–2274
17. Burandt E, Schreiber M, Stein A et al (2014) Continuous tissue microarray based identification of cancers with homogeneous target expression for successful targeted therapy in clinical routine practice. Genes Chromosomes Cancer 53:228–239
18. Ramanathan RK, Hwang JJ, Zamboni WC et al (2004) Low overexpression of HER-2/neu in advanced colorectal cancer limits the usefulness of trastuzumab (Herceptin) and irinotecan as therapy. A phase II trial. Cancer Investig 22:858–865
19. Saunders NA, Simpson F, Thompson EW et al (2012) Role of intratumoural heterogeneity in cancer drug resistance: molecular and clinical perspectives. EMBO Mol Med 4:675–684
20. Minner S, Gärtner R, Freudenthaler F et al (2013) Marked heterogeneity of ERG expression in large primary prostate cancers. Mod Pathol 26(1):106–116
21. Simon R, Mirlacher M, Sauter G (2005) Tissue microarrays. Methods Mol Med 114:257–268
22. Rosen DG, Huang X, Deavers MT, Malpica A, Silva EG, Liu J (2004) Validation of tissue microarray technology in ovarian carcinoma. Mod Pathol 17:790–797
23. Graham AD, Faratian D, Rae F, Thomas JS (2008) Tissue microarray technology in the routine assessment of HER-2 status in invasive breast cancer: a prospective study of the use of immunohistochemistry and fluorescence in situ hybridization. Histopathology 52:847–855
24. Bhargava R, Lal P, Chen B (2004) Feasibility of using tissue microarrays for the assessment of HER-2 gene amplification by fluorescence in situ hybridization in breast carcinoma. Diagn Mol Pathol 13:213–216

25. Sauter G, Lee J, Barlett JM, Slamon DJ, Press MF (2009) Guidelines for human epidermal growth factor receptor 2 testing: biologic and methodologic considerations. J Clin Oncol 27:1323–1333
26. Fernebro E, Dictor M, Bendahl PO, Fernö M, Nilbert M (2002) Evaluation of the tissue microarray technique for immunohistochemical analysis in rectal cancer. Arch Pathol Lab Med 126: 702–705
27. Kountourakis P, Pavlakis K, Psyrri A et al (2006) Clinicopathologic significance of EGFR and Her-2/neu in colorectal adenocarcinomas. Cancer J 12:229–236
28. Nathanson DR, Culliford AT, Shia J et al (2003) HER 2/neu expression and gene amplification in colon cancer. Int J Cancer 105:796–802
29. Marx AH, Burandt EC, Choschzick M et al (2010) Heterogenous high-level HER-2 amplification in a small subset of colorectal cancers. Hum Pathol 41:1577–1585
30. McKay JA, Loane JF, Ross VG et al (2002) c-erbB-2 is not a major factor in the development of colorectal cancer. Br J Cancer 86:568–573
31. Maurer CA, Friess H, Kretschmann B et al (1998) Increased expression of erbB3 in colorectal cancer is associated with concomitant increase in the level of erbB2. Hum Pathol 29:771–777
32. Seidal T, Balaton AJ, Battifora H (2001) Interpretation and quantification of immunostains. Am J Surg Pathol 25:1204–1207
33. Prat A, Parera M, Reyes V et al (2008) Successful treatment of pulmonary metastatic salivary ductal carcinoma with trastuzumab-based therapy. Head Neck 30:680–683
34. Cappuzzo F, Bemis L, Varella-Garcia M (2006) HER2 mutation and response to trastuzumab therapy in non-small-cell lung cancer. N Engl J Med 354:2619–2621
35. Santin AD, Bellone S, Roman JJ, McKenney JK, Pecorelli S (2008) Trastuzumab treatment in patients with advanced or recurrent endometrial carcinoma overexpressing HER2/neu. Int J Gynaecol Obstet 102:128–131
36. Karam A, Berek JS, Stenson A, Rao J, Dorigo O (2008) HER-2/neu targeting for recurrent vulvar Paget's disease A case report and literature review. Gynecol Oncol 111:568–571