Genetic Profiling of Cancers of the Digestive System: Biological Insights and Clinical Implications

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Abstract

Massive parallel sequencing technologies (next-generation sequencing) have enabled us to draw a comprehensive landscape of the genomic aberrations underlying common cancers of the digestive system, and they have thus revolutionized our understanding of the genomic makeup and biology of these tumors. Apart from the commonly mutated founder genes, e.g., *KRAS* and *TP53*, we now have detailed information on additional and less frequent genomic events for every major digestive system cancer. However, many challenging issues remain when it comes to translating these findings into clinical applications. Recent examples are the precise definition of the role of genomic heterogeneity and tumor evolution in metastatic spread and their impact on oncologic therapy. Other unresolved issues include the usefulness of identified drivers as novel drug targets and predictive biomarkers, as well as the development of strategies to implement broad genomic testing in individualized patient care. This review aims to dissect and discuss these topics for selected major cancers.

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Introduction

Carcinomas of the digestive system include a substantial variety of neoplasms ranging from esophageal cancer, gastric cancer (GC), small- and large-bowel cancers, and cancers of the anal region to cancers of the pancreas, liver, and bile duct system [1]. The overwhelming majority of these neoplasms are carcinomas (>90%). Epidemiologically, overall more than 3.2 million cancer deaths per year worldwide can be attributed to these neoplasms. Digestive system malignancies account for approximately 40% of cancer-related deaths in both women and men worldwide and belong to a class of cancers that have a substantial impact in developed as well as developing countries [2]. This review briefly highlights the genetic underpinnings and clinical implications of these major cancer types and discusses biological concepts in and across these tumor entities.

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The Genetic Landscape of Gastrointestinal Cancers

Colorectal Cancer

Spearheaded by Vogelstein and colleagues [3–5], accumulating work on the genetics and genomics of colorectal cancer (CRC) has revealed a biological classification into 4 major molecular subtypes [6]: (1) nonhypermethylated (<8.24 mutations per 10^6 bases, median: 58 nonsynonymous mutations) cancers with chromosomal instability (CIN) that are largely driven by mutations in *KRAS* as well as loss of heterozygosity in the tumor suppressor genes *APC* and *TP53*; (2) hypermutated (>12 mutations per 10^6 bases, median: 728 nonsynonymous mutations) CRC comprising cancers with defective mismatch repair genes (either sporadic – often in conjunction with mutated *BRAF* – or caused by germline events) resulting in microsatellite instability (MSI) and tumors with mutations mainly affecting the proofreading domain of the DNA polymerase *POLE*; (3) CRC characterized by the CpG island methylator phenotype (CIMP) showing a considerable overlap with microsatellite instable cancers and some nonhypermethylated CRC; and (4) hypermutated and nonhypermethylated cancers with genetic alterations in tetranucleotide repeats of microsatellites. These 4 groups are reflected by different morphological characteristics and clinical features. This holds particularly true for hypermutated CRC which present with a distinct phenotype (proximal colon, high tumor grade, mucinous differentiation, and strong immune response) and are associated with comparably good survival [7]. While these tumors appear to respond less well to 5-FU regimens [8], there is evidence that at least patients with MSI tumors benefit from novel immune modulatory drugs targeting the immune checkpoint response (for further details, see below) [9].

Interestingly, the mutational profile between nonhypermethylated and hypermutated CRC is quite distinct except for *APC*, which appears to be an important gatekeeper in both cancer types and highlights the important role of Wnt signaling for these cancers. While, besides *KRAS* (codons 12, 13, and 61) and *TP53* mutations, nonhypermethylated cases show frequent mutations in *NRAS*, another genetic component of MAPK signaling, and in *PIK3CA* as well as in genes encoding TGF- β signaling components (e.g., *ACVR1B*, *SMAD2*, and *SMAD4*), hypermutated cases are founded on genetic alterations of mismatch repair genes. In this context, biallelic hypermethylation of *MLH1* is accountable for most sporadic cases [10, 11] and, as *MLH1* can be one of the major methylation targets, there is a considerable overlap with CIMP-driven

tumors [12]. Sporadic MSI-high tumors seem to accumulate mutations in genes containing intrinsic coding microsatellites (e.g., *ACVR2*, *TGFBR2*, *MSH3*, and *MSH6*) where especially frameshift mutations could lead to truncated proteins that potentially serve as neoantigens for the immune system. Interestingly, though it does not harbor coding microsatellites, *BRAF* is also a frequent target for mutations in sporadic MSI-H CRC. Missense and nonsense mutations affecting *POLE* [13] can lead to hypermutated tumors as well, which are, however, microsatellite stable (MSS).

In our view, probably the most intriguing finding of the major genetic and genomic profiling and functional studies is that – at least in sporadic CRC, which account for approximately 75% of all CRC – only very few genetic alterations are required to initiate and develop CRC (founder genes) and it is assumed that many of the other mutated genes are largely passengers that tag an individual CRC as a unique tumor but do not contribute much in terms of tumor biology [14, 15]. However, we would like to point out that to our knowledge there is neither clinical nor experimental evidence that single rarely mutated genes do not contribute to shaping the biology of individual tumors. Hence, additional work is required to fully understand the functional contribution of the long tail of mutated genes in an individual context. Another important aspect we would like to highlight briefly in this context is derived from the discovery of these founder genes: as the term implies, these mutated genes are already apparent early in the development of colorectal neoplasms including clinically benign precursor lesions (e.g., frequent *BRAF* mutations in preinvasive lesions of the serrated pathway including hyperplastic polyps [16]) so that detection of a mutated gene alone – without taking the biological and morphological context into consideration – is noninformative and could be heavily misleading in a clinical scenario if diagnostic and therapeutic decisions were solely based on this single parameter.

Esophageal Cancer

Esophageal squamous cell carcinoma (ESCC) has been the dominant esophageal cancer subtype worldwide for decades, but the incidence of esophageal adenocarcinoma (EAC) has increased over the last decades in Western countries. While these two cancer types require different oncological therapies and can be clinically challenging especially with advanced disease, the knowledge on the genetic aberrations underlying these tumors has been rather limited until recently. In 2013, besides identifying mutations in the tumor suppressors *TP53* and *CDKN2A* as

well as in *SMAD4*, *ARID1A*, and *PIK3CA* that had already been implicated in EAC earlier, Dulak et al. [17] reported on additional frequently mutated genes, including the chromatin-remodeling factors *ARID1A*, *SMARCA4*, *ARID2*, *PBRM1*, and *JARID2*, some of which had already been implicated in other major GI cancers. Moreover, they additionally identified mutations in genes that appear to modulate the invasion and motility of cancer cells. Among them *ELMO1* and *DOCK2*, both of which encode dimerization partners and intracellular mediators of Rho family GTPases, were the most frequently mutated genes. Two additional genes – *SPG20* and *TLR4* – that are functionally not well characterized yet but appear to play a role in tumorigenesis were also identified as significantly mutated genes. Other recurrently mutated genes included *AKAP6*, the E3 ubiquitin ligase *HECW1*, and *AJAP1*, a gene mediating signaling at adherens junctions. Probably one of the most intriguing findings of a follow-up study conducted by Weaver et al. [18] was that even non-dysplastic Barrett's esophagus (BE; established precursor lesion of EAC) showed mutations in cancer-associated genes, e.g., *ARID1A* and *SMARCA4*, while mutated *TP53* and *SMAD4* were mainly confined to BE with high-grade intraepithelial neoplasia. This study, which is conceptually based on previous single-gene studies investigating mutations in *TP53* [19, 20], not only corroborates the role of mutant *TP53* during neoplastic progression but it also extends the current knowledge of genetic lesions in BE with biological as well as putative diagnostic and clinical implications for the future. Hence, interrogation of these genes might be suitable to supplement conventional histology in the assessment of risk progression. Complementing these studies, Agrawal et al. [21], by comparing the genetic make-up of EAC and ESCC, showed that *TP53* is the most commonly mutated gene in both cancer types and detected a considerable number of mutations in *NOTCH1*, *NOTCH2*, *NOTCH3*, and *FBXW7* that were prevalent in ESCC. Three additional studies focusing on ESCC [22–24] confirmed the high prevalence of mutated *TP53* and *NOTCH1*. The data regarding *NOTCH1* point to the fact that this gene likely plays a role as a tumor suppressor in ESCC, which is consistent with sequencing data of squamous cell carcinoma of the head and neck [25, 26], where *NOTCH1* appears to play a similar role. Other major genetic themes in ESCC comprise frequent inactivation of cell cycle regulators (*CDKN2A*, *CCND1*, *RB1*, and *NFE2L2*) as well as mutations in histone modifiers (e.g., *ASHL1*, *MLL2*, *MLL3*, and *SETD1*) and genetic alterations affecting MAPK signaling, with mutated *PIK3CA* being the most prevalent event. Another recent

large-scale profiling study from the Cancer Genome Atlas Research Network in principle confirmed these mutational profiles and additionally emphasized that both EAC as well as ESCC harbor frequent amplifications albeit with a differing profile. While *CCND1* as well as *SOX2* and *TP63* amplifications were prevalent in ESCC, EAC were prone to develop *ERBB2*, *VEGFA*, *GATA4*, and *GATA6* amplifications in a considerable number of cases. This study also established three molecular distinct subgroups for ESCC and proved that EAC molecularly profiles very much resemble that of the CIN subtype in GC (see below) [27].

Gastric Cancer

Over the last decades, a number of mutated genes in GC have been identified, including *APC*, *ATM*, *CDH1*, *CREBBP*, *CTNBN1*, *KMT2D*, *KRAS*, *TRRAP*, *MLL*, *MLL3*, *PIK3CA*, *SETBP1*, and *TP53*, among others. The first whole exome sequencing (WES) study [28] on GC confirmed many of these data and additionally identified frequent inactivating mutations in *ARID1A* encoding a member of the SWI-SNF chromatin remodeling family. Mutations frequencies varied according to biological subtypes, with *ARID1A* mutations being most prevalent in MSI-driven tumors, closely followed by tumors with EBV infections. Following up on this work, Zhang et al. [29] provided supportive results for a crucial role of *ARID1A* in GC carcinogenesis and additionally identified mutations and deletions in *FAT4*. The authors concluded that these two genes may act as important tumor suppressors that become inactivated during GC carcinogenesis. Moreover, similar to hepatocellular carcinoma (HCC) (see below), they detected mutations in two other chromatin remodeling genes, i.e., *MLL* and *MLL3*. A year earlier, this group had already shown [30] recurrent mutations in *MAP2K4*, and gene fusions involving *CDK12* and *ERBB2* in GC. Employing whole genome sequencing (WGS), Wang et al. [31] refined the mutational landscape by adding *MUC6*, *CTNNA2*, *GLI3*, *RNF43*, and *RHOA* to the set of mutated genes in GC. Interestingly, mutations in the latter gene were specifically observed in diffuse-type but not in intestinal-type GC. Wong et al. [32] identified further mutations in genes encoding ephrins and components of the SLIT/ROBO signaling pathway.

Comprehensively summarizing and extending the previous work briefly highlighted above, the Cancer Genome Atlas Research Network [33] suggested dividing GC into 4 distinct biological subtypes: EBV-infected tumors, MSI-driven tumors, genomically stable tumors, and tumors with a CIN phenotype. EBV GC harbor a spe-

cific transcriptional profile, which is modulated by methylation of the promotor regions of various tumor suppressors, with *CDKN2A* almost always being affected. Moreover, this tumor subtype is strongly enriched for recurrent *PIK3CA* mutations (80%) as well as for mutations in *ARID1A* (55%) and *BCOR* (20%) encoding for an antiapoptotic protein, while mutations in *TP53* were rarely observed. Interestingly, these tumors also harbor recurrent amplifications of *ERBB2* and genes located at chromosome 9p (*JAK2*, *PD-L1*, and *PD-L2*). In contrast, MSI tumors show elevated mutation rates and a higher rate of mutations in genes encoding oncogenic signaling proteins (*ERBB3*, *PIK3CA*, *KRAS*, *NRAS*, and *PTEN*). Interestingly, however, this tumor subtype did not harbor *BRAF* mutations that are frequently observed in sporadic MSI-driven CRC. The third group, i.e., genomically stable GC, displayed mutations in *RHOA* or fusions involving RHO family GTPase-activating proteins and was enriched for the diffuse histological variant. Lastly, tumors with CIN showed high mutation rates in *TP53* accompanied by marked aneuploidy with focal amplification of receptor tyrosine kinases and cell cycle regulators (*VEGFA*, *CCNE1*, *CCND1*, and *CDK6*).

Finally, recent work from Cristescu et al. [34] that was mainly based on transcriptional profiling but also employed targeted sequencing and SNP arrays described 4 molecular subtypes that correlated with clinicopathological parameters including survival. Briefly, they identified 2 major classes of GC: MSI-driven tumors and MSS tumors. MSS tumors were further classified according to *TP53* activity status and the presence/absence of an EMT signature. Applying this classifier to their cohort, they found that patients harboring GC of the MSI subtype had the best prognosis, while MSS tumors showing the EMT signature were associated with a dismal survival. They were able to substantiate their results by utilizing data of 3 independent studies for validation. Taken together, these data indicate that, while all of these studies provided great insight into the genetics of GC tumors, we now have to funnel resources to precisely understand the true biological impact of these findings and translate the results into novel therapies that benefit patients.

Hepatocellular Carcinoma

Over the last couple of years several major studies [35–39] have confirmed long-known but also identified new genetic themes in HCC. Among them, recurrent mutations in *CTNNB1* and *AXIN1*, as well as in *CDKN2A*, *TP53*, *ATM*, and *IRF2* indicate that Wnt/ β -catenin signaling and impaired cell cycle regulation are

the main drivers of HCC carcinogenesis. Other emerging themes are functional perturbation of chromatin remodeling genes (*ARID1A* and *ARID2*), recurrently mutated genes involved in acetylation and methylation of nucleosomes (*MLL*, *MLL2*, *MLL3*, *MLL4*), and functional activation of the *NFE2L2/KEAP1*-mediated signaling cascade, respectively.

In addition, activating mutations in *PIK3CA* and inactivating mutations of *RPS6KA3* point to a role for PI3K/Akt/mTOR signaling at least in some HCC. As reported by Kan et al. [38], a subset HCC with underlying HBV infection harbor activating mutations of *JAK1* (9%), and Nault et al. [40] identified frequent mutations (59%) in the promotor region of *TERT* that confer increased activity to telomerase reverse transcriptase. Interestingly, the same group very recently showed clonal integration of adeno-associated virus type 2 (AAV2) in known driver genes [41], such as *CCNA2*, *CCNE1*, and *TERT*, among others, opening up another chapter of our understanding of HCC development. These data are in line with reports describing HBV insertions in *TERT* and *MLL4*, respectively [42, 43]. While the precise functional role of many of these genetic aberrations and in particular for mutated genes that execute epigenetic regulation, as well as for alterations in the NRF2/KEAP1 pathway involved in the cellular oxidative stress response, remains to be explored in the next years, these data provide important insights into HCC carcinogenesis that will hopefully be clinically exploitable in the near future. In this context, the aforementioned studies and in addition the recent discovery of unique and highly recurrent *DNAJB1-PRKACA* fusions in fibrolamellar HCC [44] convey an important message: it is crucial to consider the different etiologies of HCC and distinct phenotypes/rare subtypes to obtain a truly comprehensive and detailed landscape of genetic aberrations and their associations with clinical parameters. This applies to HCC in particular but can be deduced as a general concept to which all cancer profiling studies should ideally adhere.

Cholangiocarcinoma

Besides mutations in canonical genes implicated in other malignancies of the digestive system (*KRAS*, *TP53*, and *CDKN2A*), a recent study investigating intrahepatic cholangiocarcinoma (ICC) detected additional mutations in genes belonging to the family of chromatin-modifying genes (*ARID1A*, *BAP1*, and *PBRM1*; 10–20%) [45]. In addition, confirming previous studies, frequent mutations in *IDH1* and *IDH2* (20%) were detected in the same group of tumors, and the discovery of mutated *FGFR2* matches more recent data describing recurrent *FGFR2-PPH1N1*

fusions [46]. As discussed further below, these genetic aberrations appear to be clinically exploitable. The latter study also described mutations in the *ARAF* oncogene, whose precise function in this biological context remains to be explored. Both studies reported recurrent mutations in genes encoding PIK3-AKT and MAPK signaling (*KRAS*, *NRAS*, *PIK3CA*, *PIK3C2G*, *PIK3C2A*, and *PTEN*), underscoring the importance of these pathways in the development of cholangiocarcinoma (CC). Jiao et al. [45] investigated 2 very small sets of gallbladder cancers and found mutated *TP53* to be the most prevalent genetic aberration, followed by mutations in the chromatin-remodeling genes *ARID1A*, *BAP1*, *MLL3*, and *PBRM1*. While the latter findings appear to recapitulate the genetic landscape of HCC and CC of the bile ducts, the high frequency of *TP53* mutations may point to the fact that, while there are common genetic themes shared across these cancer subtypes, clearly distinct genetic aberrations may propel and contribute to the different biological behavior. In addition, 2 studies investigating fluke-related CC [47, 48] identified a particularly high rate of *TP53* mutations in these tumors and, by comparing these sequencing data with non-fluke-related CC, Chan-On et al. [47] concluded that these two tumor types show a reciprocal mutational pattern whereby mutations in chromatin modifiers as well as in *IDH1* and *IDH2* were much more prevalent in non-fluke-related tumors while *TP53* mutations were much more common in fluke-related CC. These results emphasize that the analysis of cancer subtypes including different etiological contexts is paramount to truly understand the genetic basis of apparently homogenous tumor entities, and further genetic studies comprehensively dissecting CC subtypes are warranted.

Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is not only one of the deadliest cancer types but also still one of the most enigmatic malignancies for which novel effective treatment approaches are crucially required. With the data of recent molecular profiling efforts at hand, there is now a strong basis for future endeavors aimed at understanding the interplay of driver pathways and at developing drugs that will ultimately help to keep PDAC biology in check. In 2008, the pioneering work by Jones et al. [49] demonstrated a high prevalence of mutations in genes encoding *KRAS* signaling [50] which was in line with previous studies showing that mutated *KRAS* is a founder gene for PDAC that is already present in premalignant dysplastic lesions [51–55]. Besides *KRAS*, frequently mutated genes include *TP53*, *CDKN2A*, and *SMAD4* – all well-

known drivers of other major cancers of the digestive system. There is, however, a long tail of infrequently mutated genes contributing to the 12 core signaling pathways that were identified as active in PDAC by Jones et al. [49]. Subsequent work on metastatic PDAC [56, 57] identified a certain degree of genetic heterogeneity and subclonal branches that could be traced in the primary and metastatic lesions. Moreover, structural rearrangements (“fold back inversions”) were identified that appear to be early events in PDAC development and may contribute to amplifications in the PDAC genome. In 2012, Biankin et al. [58] confirmed the high prevalence of mutations in *KRAS* as well as in the other canonical genes and also corroborated the presence of mutations across many genes at low frequencies. Interestingly, similar to HCC, they also detected mutations in genes that control epigenetic regulation of the genome (e.g., *MLL3*, *ARID1A*, *EPC1*, and *ARID2*), as well as in the DNA damage repair gene *ATM*. Moreover, they showed mutations in 5 genes whose functions in PDAC carcinogenesis are not well understood to date (*ZIM2*, *MAP2K4*, *NALCN*, *SLC16A4*, and *MAGEA6*). Spearheaded by Alexandrov et al. [59], analysis of mutational signatures across PDAC identified additional major mutagenic themes including activation of the APOBEC family of deaminases, *BRCA*-mediated DNA maintenance deficiency, and DNA mismatch repair deficiency. With additional efforts refining the aforementioned data [60–62], probably less than 10 major genetically perturbed pathways emerge that mainly contribute to the origin and development of PDAC and may be targetable to some extent: MAPK signaling, TGF- β and Wnt signaling, cell cycle regulation, DNA damage response, and chromatin remodeling, as well as MYC- and NOTCH-driven signaling cascades.

The Evolution of Clinical Genetic Testing in Tumors of the Digestive System

After having briefly summarized the molecular landscapes of cancers of the digestive system (Fig. 1) and the tremendous impact of massive parallel sequencing (MPS) on our understanding of the genetic landscape and biology of these neoplasms, the second part of this review will focus on the implications of these novel insights for the clinical management of patients with cancers of the digestive system.

Among solid tumors including cancers of the digestive system, specifically CRC serves as a role model for the successful implementation of a specific genetic test that

Fig. 1. Common molecular themes in cancers of the digestive system. Common frequently mutated driver genes across tumors of the gastrointestinal tract and the hepatopancreatic system.

governs treatment decisions. This pioneering work was triggered by observations that were made more than 10 years ago showing that patients with *KRAS* mutant CRC do not respond to EGFR targeting agents such as the monoclonal antibodies cetuximab [63, 64] and panitumumab [65]. These data subsequently led to a major change in the approval of these drugs by the European Medical Agency (EMA), which in 2008 demanded genetic testing of CRC for the presence of *KRAS* mutations as a prerequisite for antibody treatment. The reason for this decision was to yield a high level of patient benefit by balancing treatment efficacy and unwanted side effects. Of course, the dictum of the EMA also kept an eye on the development of health care costs, with molecular stratification influencing cost-benefit ratios in medical oncology. The overall success of this stratification approach, which can well be viewed as the advent of genetically informed personalized medicine in medical oncology, subsequently led to a refinement of this strategy via the addi-

tion of less frequent mutations in other *RAS* genes to the panel of negative response predictors in the anti-EGFR setting [66]. In addition, mutations in other genes encoding downstream signal transducers of the MAPK cascade, such as *BRAF*, also appear to have a negative predictive value in MSS tumors [66]. These discoveries led to a change in the clinical guidelines for genetic testing, which now include a broad portfolio of alterations in *RAS/RAF* genes. Very recently, an analysis of several trials revealed that patients with *KRAS*-wildtype tumors show a different outcome when stratified into left-sided and right-sided tumors. Right-sided cancers are associated with a comparably poor prognosis, and it remains to be explored which molecular features govern the phenomenon of “sidedness” in CRC [67]. The second clinically established molecular stratifier in gastrointestinal malignancies constitutes Her2 expression as a positive response predictor for anti-ErbB2 drugs (such as trastuzumab) in GC [68]. Although measurement of expression levels of

Fig. 2. Predictive molecular biomarkers in cancers of the digestive system. Current status of established (a) and potential future (b) molecular predictors in these cancers.

specific therapeutic targets by MPS technologies is not common practice for routine diagnostics yet, this situation may change in the near future when RNA sequencing techniques using formalin-fixed paraffin-embedded specimens become more widely available [69].

That being said, one could argue that while the application of MPS technologies is certainly interesting from a scientific point of view, there are still some obstacles on the road to clinical implementation. At first glance, a broad genomic characterization of cancers of the digestive system in the clinical setting may appear like cracking nuts with a sledgehammer as right now only *RAS* mutations in CRC are truly established response predictors (Fig. 2). However, the field is moving fast and new discoveries are being made at an accelerating speed [70]. First, a multitude of clinical studies are underway which are aimed at codiscovering predictive genetic targets together with effective novel targeted drug combinations [71] (see below) in almost all cancers of the digestive system. If only a fractal of these studies is successful, the current test approach will broaden tremendously with respect to both specific markers and the amount and types of cancers that require testing. Second, with prices for MPS continuously dropping, even for *RAS/RAF* testing the cost-effectiveness turning point from conventional Sanger techniques to MPS is about to be reached [72]. Third, multigene test-

ing for deselection of patients who will not benefit from a specific therapy has been shown to be cost-effective even in the setting of high test costs [73]. Finally, nowadays a variety of novel precision medicine approaches specifically aim to broadly stratifying patients to as yet nonapproved drugs/drug combinations as a final rescue attempt [74]. This applies in particular to last-line therapy settings in common tumor entities and to patients with rare tumors without firmly established therapy regimens.

Genetic Heterogeneity of Tumors of the Digestive System and Its Clinical Implications

In 1976, Peter Nowell, a pathologist and codiscoverer of the Philadelphia chromosome, published theoretical considerations on clonal evolution of cancers [75] which proved to be so farsighted and ahead of the curve that it took more than 30 years, and the evolution of several new technologies, for several groups to start to follow up on this concept experimentally and clearly demonstrate that cancers including solid malignancies are indeed composed of more than one cell clone. In 2008, Campbell et al. [76] provided evidence of subclonal diversity on the genetic level in B-CLL, and only 2 years later the

same group [56] reported on the same phenomenon in PDAC with the demonstration of site-specific tumor cell branches. Similar data for PDAC were shown by Yachida et al. [57] the same year. Adding to this pioneering work, in 2012 Gerlinger et al. [77] contributed to the concept of genetic heterogeneity of tumors by demonstrating spatiotemporal genetic differences in clear cell renal cell cancer. Based on these landmark papers, the existence of competing cellular subclones and clonal evolution as measured by next-generation sequencing has emerged as an important topic in cancer research over the last couple of years. Several groups have even suggested that genetic tumor heterogeneity might explain why the success stories of biomarker discovery and identification of drug targets are modestly small to date [78–80]. The emerging picture is now that almost every major cancer of the digestive system, including CRC [81], PDAC [56], esophageal carcinoma and its precursors [82, 83], CC [84], and HCC [85], is composed of more than one tumor cell clone. The degree of genetic heterogeneity, however, appears to be quite variable. While the concept of tumor heterogeneity and the putative implications for tumor biology and therapy are exiting avenues of research worth following up on, and recent single-cell approaches will provide even more detailed insights into clonal diversity, in our view several open questions remain. First, most studies equate heterogeneity with genetic heterogeneity by definition, thereby largely ignoring the assessment of other molecular levels within tumor cells (e.g., proteome, transcriptome, and methylome), which could potentially contribute to tumor diversity. Second, based on these considerations, it becomes readily apparent that the heterogeneity of a tumor can be investigated on many levels [86] also including the histological phenotype and at various degrees of resolution which may lead to different or even divergent results according to the parameters analyzed. Third, although the evidence of genetic heterogeneity in cancers is compelling, the true functional consequences of many findings in terms of tumor biology and clinical behavior remain rather unclear. This applies in particular to genes that are mutated at low frequencies and whose functions including those of their wild-type counterparts remain to be discovered, but it also holds true for mutated major driver genes whose function and biological impact appear to be different according to their biological (genetic) context [70, 87–90]. Fourth, most studies including clinical trials have shown that founder mutations, which serve as prime targets for therapy or predictive biomarkers, are

stable in primary tumors and their metastatic sites [91–93]. In this context, a very recent perspective by Vogelstein and Kinzler [15] pointed out that a certain level of (genetic) heterogeneity will be present even in every normal tissue that is investigated and the really intriguing issue about cancer is possibly its peculiar genetic homogeneity rather than its heterogeneity. As pathologists, we encounter cases with varying degrees of tumor heterogeneity on a phenotypic level almost every day while viewing slides and signing out cases, and therefore we readily acknowledge the general concept of tumor evolution. The true biological and clinical significance of genetic heterogeneity for every specific tumor type, however, remains to be meticulously dissected and determined to avoid premature and possibly even misleading conclusions.

Novel Modalities for Genomic Testing and Their Role in Tumors of the Digestive System

With the first human genomes having been sequenced only about 15 years ago [94], it did not take long until the introduction of predictive genetic testing in routine tumor diagnostics [65]. Since then, an unprecedented development has taken place that has the potential to revolutionize oncology and pathology as we now know it. In the early days of genomic predictive tumor testing, the choice of methods was mainly among Sanger sequencing technologies, pyrosequencing, and allele-specific PCR methods, as exemplified for CRC [95]. However, when oligogene testing in lung cancer became the standard of molecular diagnostics about 5 years ago, molecular pathology laboratories began to explore the application of MPS technologies for genetic profiling of cancers in general using formalin-fixed paraffin-embedded material obtained from routine diagnostics. Initially many institutions focused on customized multigene panels [96–99] either with a one-fits-all approach or in an entity-tailored fashion as sequencing of whole exomes or even whole genomes in high-throughput routine diagnostics was (and still is) neither cost-effective nor efficient in terms of turnaround time. Additionally, technical difficulties with handling formalin-fixed paraffin-embedded and low-input amounts of DNA hampered broader sequencing approaches until now. However, with the advent of more sophisticated technologies that allow RNA and DNA sequencing even at single-cell resolution [100] and dropping costs, WES and WGS may become more widely available in routine diagnostic focus applications over

time [101]. The big advantage of these approaches is that holding available specific up-to-date gene panels for different purposes and constant gene panel modifications and adoptions due to new discoveries would not be necessary anymore. Moreover, WES and WGS data could potentially be used to make true unbiased discoveries during routine diagnostics by comprehensive (pro- or retrospective) analysis of data sets. This applies not only to the discovery of the true prevalence of predictive and less frequent genetic aberrations in the own intention-to-treat population but also to the detection of novel mutational patterns in subsets of major cancers as well as in rare tumors that may reshape diagnostic categories or will be clinically exploitable. Those data are needed from many centers on a national level and more ideally from around the world to establish a comprehensive database that provides an overview of mutational aberrations across all kinds of tumors and ideally links them to therapy response data [102]. While to some extent discovery by routine diagnostics can also be achieved by current targeted gene panels [96], this approach is certainly less powerful compared to WES/WGS. However, apart from technical considerations, the major challenge of all broad one-size-fits-all approaches is implementing a bioinformatics pipeline that obtains meaningful and valid results from WES and WGS data for routine diagnostics with a low turnaround time and at affordable costs.

That being said, it is nowadays evident that targeted sequencing approaches outperform conventional sequencing techniques in routine diagnostic molecular pathology [103]. In addition to the detection of simple somatic mutations, the technology can also be used to detect genetic macroaberrations such as copy number variations [104] and gene fusions [69, 105] in routine diagnostics. With the technical issues regarding the implementation of targeted MPS in diagnostics being resolved, in our view issues of quality assurance [106] as well as reimbursement mark the last steps on the way to a broad use in routine molecular diagnostics across pathology centers.

Novel Targetable Genetic Lesions in Cancers of the Digestive System

Leaving molecular profiling for clinical trials aside [107], current molecular stratification approaches for the daily practice of cancer precision medicine mainly follow two roads. The first approach aims for a comprehensive molecular stratification of an individual tumor (e.g., in-

cluding exomic/genomic, transcriptomic, and epigenomic data) and the subsequent application of individually selected – mostly off-label – therapies. However, these efforts are restricted to programs of selected major comprehensive cancer centers, and a broad application in routine diagnostics appears to not be feasible right now. A major challenge that lies ahead is that success stories derived from the current “individual patient” approach will likely remain on an anecdotal level unless large databases for cross-comparison of many individual data sets are established in order to fully understand the meaning of the clinical results.

The second approach aims for a step-by-step inclusion of novel single or oligomarker predictors for defined therapeutics in an entity-stratified manner. These marker-drug combinations have already been validated in a clinical study context and they have gained approval from the regulatory authorities. While a multitude of novel targeted drugs/marker combinations are currently being tested, Figure 2 provides a selection of the most relevant targets/markers for cancers of the digestive system that can be expected to enter the clinical arena in the next 2 years and which we will discuss in more detail below.

Colorectal Cancer

In CRC the focus in recent years has been on refinement of patient selection for anti-EGFR therapy. To this end, several novel biomarkers have been proposed, including *PIK3CA* mutations and others [66, 88, 108, 109]. A second class of drugs, which have been tested in a biomarker-selected population, comprise mutant-specific BRAF inhibitors. Although single agents or combinations with MEK inhibitors had been unsuccessful, preclinical modeling showed that combinations with EGFR inhibitors might be more promising. This led to clinical testing of this combination [87]. For another class of targeted agents – the antiangiogenic chemotherapeutics – active in CRC predictors of response have not yet been identified although major efforts have been directed toward this issue [110, 111]. Apart from targeted agents, immune checkpoint inhibitors emerged as a new class of drugs that were designed to harness the immune system in the fight against cancer. A recent landmark study showed that specifically microsatellite instable CRC respond well to this therapeutic approach [9], presumably due to a higher mutational load of these tumors, which increases the likelihood of yielding novel immunogenic neoantigens [112]. From a molecular diagnostics point of view, the MSI status of CRC will therefore likely receive new attention that goes well beyond genetic counseling and

has direct therapeutic implications. While classic PCR-based assays will do the job, it would be desirable to implement MSI testing in current MPS approaches to ensure a one-stop shop. However, the direct interrogation of repetitive genomic sequences is still a major technical challenge in this context that needs to be resolved. A broader test approach potentially identifying all tumors with high mutational load regardless of the actual underlying cause (e.g., *POLE* mutations [113, 114]) could be exome sequencing. Recent data also suggest that overall mutational load as surrogate marker for MSI status can also be inferred from panel sequencing data [115]. Another approach could be the interrogation of frameshift mutations in specific genes known to be recurrently affected in MSI tumors. However, before implementation in routine diagnostics, additional data are needed to fully understand the relation between the efficacy of immune checkpoint inhibition and the mutational burden of different cancers.

Gastric, Gastroesophageal, and Esophageal Cancer

While gastric adenocarcinomas are usually lumped together with adenocarcinomas of the esophageogastral junction (AEG) and the esophagus, squamous cell carcinomas of the esophagus clearly constitute a molecularly and clinically distinct tumor entity. While for the latter, as for most squamous cell carcinomas, no convincing molecular predictors have been discovered so far, the former tumor entities obviously represent more accessible targets for novel chemotherapeutic treatment options [116]. This has been exemplified by the success of trastuzumab in Her2-positive gastric adenocarcinomas and AEG. Her2 targeting strategies are still being explored and expanded. In addition, MET has been identified as a promising targetable molecule in AEG and gastric adenocarcinomas overexpressing the protein [117]. VEGF targeting agents, which have also been recently identified as active in AEG and gastric adenocarcinomas, still come without any specific biomarker [118]. EGFR-inhibiting drugs have been tested for efficacy specifically in esophageal cancer, albeit without groundbreaking success to date [119]. As in CRC, immune checkpoint inhibitors might be effective in GC [120], and recent data indicate that MSI-driven tumors are likely to respond to this type of treatment [121]. As PD-L1 expression has been proposed as a response predictor for therapy with immune checkpoint blockers, patient selection will initially rely on straightforward PD-L1 IHC while the precise role of MSI analysis and mutational load assays remains to be determined.

Liver Cancer and Pancreatic Cancer

As already pointed out above, liver cancer is divided into HCC and CC, which in their own right are a group of molecularly quite divergent ICC and extrahepatic CC, with the latter bearing a strong molecular resemblance to PDAC.

Unfortunately, so far almost all strategies attacking HCC with targeted drugs have failed. Only the multikinase inhibitor sorafenib, showing a modest clinical response, has gained approval but, to date, no specific molecular predictive biomarkers have been identified for this drug [122, 123]. One of the reasons for this continuous failure of targeted agents in HCC is a certain lack of drugable genes in these tumors. The two most promising drug/biomarker combinations currently being explored comprise MET inhibitors which appear to have a certain activity in MET-expressing HCC [124] and agents targeting FGF19 which was found to be amplified in a subset of HCC [125].

In principle, a similar situation exists for CC. Although we know the molecular landscape of both ICC and extrahepatic CC fairly well, none of the aberrations discovered has really emerged as a major drug target or could be used as a biomarker for treatment response so far. That being said, there may be some hope on the horizon as very recently recurrent gene fusions involving FGFR2 in ICC have been described [46], which are druggable targets. In this context, more recent data demonstrate that these tumors develop resistance mutations during treatment with FGFR inhibitors, an evolutionary phenomenon that is well known in lung cancers [126]. Another promising approach centers on the selection of CC with Her2 amplification/overexpression that appear to respond to anti-Her2 treatment [127]. While current medical concepts still rely on conventional chemotherapeutic combinations [128], these two examples point towards a new direction in CC therapy.

PDAC is a tumor that is genetically founded on almost universally present undruggable KRAS mutations and has been shown to be resistant to a multitude of targeted therapies tested thus far [129]. So far, only erlotinib has been approved as a targeted agent but the survival advantage is minimal and no biomarker selection strategies have been identified for this drug to date [130]. The most promising potentially druggable family of molecular lesions, which can be observed in a small subset of PDAC, comprise mutated *BRCA1* and *BRCA2*. Patients with mutations in these genes have been reported to respond to PARP inhibitory drugs in high-grade serous carcinoma of the ovary through a mechanism of synthetic lethality

Fig. 3. Potential biomarkers and/or druggable mutations in cancers of the digestive system projected on oncogenic pathways. Target alterations in gastrointestinal and hepatopancreatic cancers are marked in black.

[131]. Preliminary data on very small PDAC patient populations indicate that this mode of action might also be true for pancreatic cancer [132], and it may well be that BRCAness and DNA repair deficiency in general are molecular themes that can be therapeutically exploited in specific subgroups of all digestive cancer types.

Conclusion

Over the last decade a multitude of efforts have led to the description of a fairly precise molecular landscape of gastrointestinal and hepatopancreatic cancers. However, many uncertainties (e.g., the prevalence and role of low-frequency mutations, gene fusions, specific genotype-phenotype associations, and rare tumor subtypes) prevail which define the need for further confirmation and – more importantly – extension of the published data sets. Also, still too little is known about the genetic hetero-

geneity of these neoplasms and how precisely and to which extent genetic heterogeneity that is believed to fuel cancer evolution contributes to metastatic spread and relapse and interferes with current therapy approaches. Another line of research will be directed towards the genetic foundations of clinical response towards checkpoint blockers, including mutational load, MSI status, PD-L1 amplifications [133, 134], as well as putative resistance mutations [135, 136]. Today, with many major technical challenges being resolved, a multitude of cancer centers implement at least targeted MPS technologies in routine diagnostics. In our view, with an arsenal of molecular profiling tools at hand, the real bottleneck and true challenge for therapy of cancers of the digestive system is now the identification of powerful genetic drug targets and biomarker combinations that benefit patients (Fig. 3). As outlined above, some – but still too few – are about to appear on the horizon and will be hopefully the beginning of a new era in the treatment of neoplasms of the digestive system (Fig. 2).

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